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(54) Title: METHODS AND COMPOSITIONS FOR TREATMENT OF ALZHEIMER'S DISEASE BY ENHANCING PLASMIN OR PLASMIN-LIKE ACTIVITY
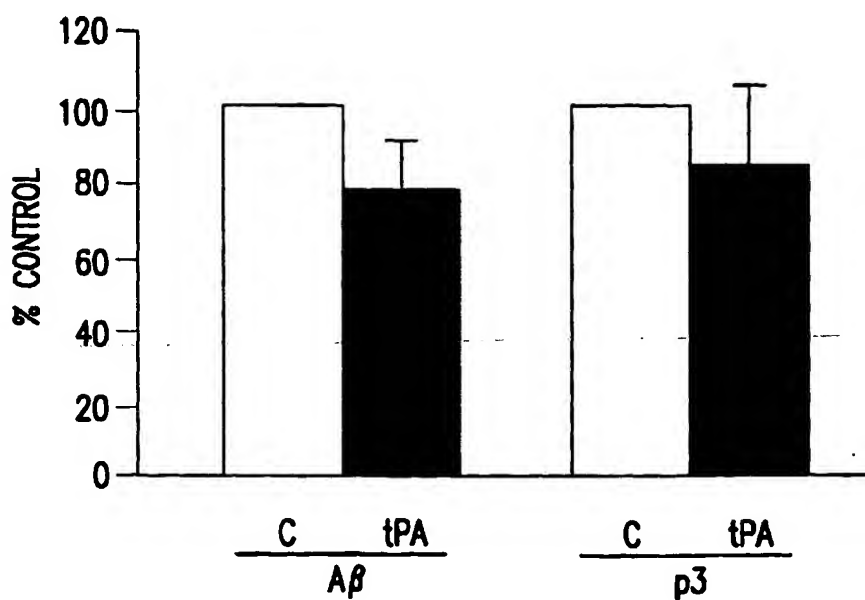
(57) Abstract: The present invention relates to methods of treating or preventing Alzheimer's disease in a subject by causing an increase in plasmin or plasmin-like activity in the subject's brain. The present invention further relates to compositions useful for increasing the plasmin or plasmin-like activity in a subject's brain, and to methods of identifying compositions useful for increasing plasmin or plasmin-like activity in a subject's brain. In addition, methods of detecting Alzheimer's disease are provided.

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METHODS AND COMPOSITIONS FOR TREATMENT OF ALZHEIMER'S DISEASE BY ENHANCING PLASMIN OR PLASMIN-LIKE ACTIVITY

1. INTRODUCTION

The present invention relates to methods of treating or preventing

5 Alzheimer's disease in a subject by causing an increase in plasmin or plasmin-like activity in the subject's brain. The present invention further relates to compositions and methods useful for increasing the plasmin or plasmin-like activity in a subject's brain. The present invention also relates to methods of identifying compositions useful for increasing plasmin or plasmin-like activity in a subject's brain.

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2. BACKGROUND OF THE INVENTION

2.1. ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a degenerative brain disorder characterized

15 clinically by a progressive loss of memory, confusion, dementia and ultimately death. Histopathologically, Alzheimer's disease is characterized by the presence in the neocortex, especially the hippocampus, of two brain lesions: the neurofibrillary tangles (NFTs) of paired helical filaments (PHF) in the neurons and the neuritic (senile) plaques in the extracellular space. The formation of senile plaques is related to the appearance of the

20 symptoms and signs of the disease, including amnesia. After the formation of senile plaques, neurofibrillary tangles are produced in the neuronal bodies. The formation of neurofibrillary tangles is related to the worsening of amnesia and other symptoms of dementia. Neurofibrillary tangles are formed by the abnormal state of phosphorylation of the cytoskeletal protein Tau.

25 A major component of the senile plaques in Alzheimer's disease is a polypeptide referred to herein as Aβ (amyloid-β). Aβ also accumulates in the wall and the lumen of the brain vessels. Aβ is normally a soluble component of the cerebrospinal fluid where it is found in concentrations of about 3-5 nM. Aβ may have 39 to 43 amino acids, typically 40 amino acids, in the mature form and is derived as a proteolytic cleavage

30 product from a cell surface protein called the amyloid precursor protein (APP) (Kang *et al.*, 1987, *Nature* 325:733-736). The normal function of Aβ is not known at present but might be to form cation selective channels across cell membranes (Kawahara *et al.*, 1997, *Biophysical Journal* 73/1, 67-75).

There are several animal models of Alzheimer's disease (Higgins, 1999,

35 *Mol. Med. Today* 5:274-275). In one model, the insertion and overexpression in mice of a mutant form of the human amyloid precursor protein gene as a minigene under the

regulation of the platelet-derived growth factor β receptor promoter element causes the appearance of a synaptic pathology and amyloid-β peptide deposition in the brain of the mice. These changes in the brains of transgenic animals are similar to those seen in human Alzheimer's disease (Games *et al.*, 1995, *Nature* 373:523-527).

5   In a similar model, transgenic mice were generated by using a promoter derived from the mouse neurofilament-light gene to drive expression in nerve cells of the mouse 42-amino acid Aβ protein (LaFerla *et al.*, 1995, *Nat. Genet.* 9:41-47). Such mice also exhibit insoluble Aβ deposition in the brain.

The major form of Alzheimer's disease is sporadic and has a late onset, whereas a small percentage of cases are familial and have an early onset. Some of the

10 familial cases of Alzheimer's disease are strongly associated with one or more mutations at different sites on the Aβ precursor protein, the gene of which lies on chromosome 21. Whether these mutations are the cause of Alzheimer's disease in the affected patients, however, has not been proven experimentally.

The plaques are not unique to Alzheimer's disease. The senile plaques are

15 also seen in Down syndrome and in both aged human and animal brains. The numbers of plaques in non-demented aged humans are sometimes similar to those seen in Alzheimer's disease cases (Katzman *et al.*, 1988, *Ann. Neurol.* 23:138-144).

Amyloid plaques are the consequence of an increased production and aggregation of βA4, the 4kD fragment of the amyloid precursor protein (APP). APP is a

20 single transmembrane spanning domain protein which undergoes different proteolytic cuts during transport along the secretory pathway and on the plasma membrane. An uncharacterized protease cleaves at a site known as α-secretase site (position 687 of the N-terminus). This cleavage releases a large extracellular fragment (secretory APP) and after a second cleavage at the γ-secretase site (position 711 or 713) a small 3 kD peptide known as

25 p3 is produced. An alternative processing may occur by the action of a secretase at the β-secretase site, located a few amino acids towards the amino-terminus of the α-secretase site (position 671). Cleavage at both the β-secretase site and the γ-secretase site produces the 4kD amyloidogenic fragment βA4, also known as Aβ. Although absolute measurements have not been made, it is known that normally APP is more often cleaved at the α-secretase

30 site than it is at the β-secretase site. This suggests that processing of APP under physiological conditions favors the formation of p3 over the formation of Aβ. Since p3 is non amyloidogenic and cleavage at the α-secretase site prevents formation of the amyloidogenic βA4, cleavage of APP at the α-secretase is preferable to cleavage at the β-secretase site.

35 In patients suffering from early onset or familial Alzheimer's disease, a genetic defect is responsible for the higher amounts of βA4. The main causes are either

increased susceptibility to proteases at or around the β-secretase site of APP or increased γ-protease site cleavage activity. Although the number of patients with these defects is very small compared with the rest of the population with Alzheimer's disease (less than 5%), the findings in the genetic defect-associated cases of Alzheimer's disease led to the assumption that the presence of βA4 in the brains of subjects suffering from Alzheimer's disease

5   reflects an increased protease activity at the β-secretase and γ-secretase sites.

2.2.   PLASMIN AND PLASMINOGEN

Plasmin, a serine protease that circulates in the body as the inactive proenzyme, plasminogen, is involved in the degradation of fibrin clots. Any free circulating

10   plasmin is rapidly inhibited by α2-antiplasmin. Plasminogen binds to both fibrinogen and fibrin, thereby being incorporated into a clot as it is formed. Tissue plasminogen activator (tPA) and, to a lesser degree, urokinase plasminogen activator (uPA) are serine proteases which convert plasminogen to plasmin. Inactive tPA is released from vascular endothelial cells following injury; it binds to fibrin and is consequently activated. uPA is produced as

15   the precursor, prourokinase by epithelial cells lining excretory ducts. The role of urokinase is to activate the dissolution of fibrin clots that may be deposited in these ducts.

Active tPA cleaves plasminogen to plasmin which then digests the fibrin; the result is soluble degradation product to which neither plasmin nor plasminogen can bind. Following their release, plasminogen and plasmin are rapidly inactivated by their

20   respective inhibitors. The inhibition of tPA activity results from binding to specific inhibitory proteins. At least four distinct inhibitors have been identified. Of these, plasminogen activator-inhibitors type 1 (PAI-1) and type 2 (PAI-2) are of the greatest physiological significance. The plasminogen activators also are useful for controlling coagulation. Because tPA is highly selective for the degradation of fibrin in clots, it is

25   extremely useful in restoring the patency of the coronary arteries following thrombosis, in particular during the short period following myocardial infarct. Streptokinase (an enzyme from the Streptococci bacterium) is another plasminogen activator useful from a therapeutic standpoint. However, it is less selective than tPA, being able to activate circulating plasminogen as well as that bound to a fibrin clot.

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2.3.   ENOLASE

During glycolysis, α-enolase catalyzes the conversion 2-phosphoglycerate to phosphoenoylpyruvate. Neuron specific enolase, also known as γ-enolase, is one of the five known isozymes of the glycolytic enolase. This enzyme is released into the CSF when

35   neural tissue is injured. Neoplasms derived from neural or neuroendocrine tissue may release NSE into the blood (Johnson et al., 1984, Cancer Res. 44:5409-14). Enolase on the

surface of cells is capable of binding plasminogen, thereby localizing the plasminogen to the extracellular surface.

Citation or discussion of a reference hereinabove shall not be construed as an admission that such is prior art to the present invention.

5   3.   **SUMMARY OF THE INVENTION**

The present invention relates to methods of treating or preventing Alzheimer's disease in a subject by causing an increase in plasmin or plasmin-like activity in the subject's brain. Such methods include administration alone or in combination of compositions having plasmin or plasmin-like activity, such as plasmin, plasminogen,
10   chimeric plasminogen; the administration of activators of plasmin, such as tissue-type plasminogen activator, urokinase-type plasminogen activator, α-enolase, staphylokinase, aspirin; the administration of agents able to enhance activator activity or concentration, such as sulodexide, recombinant brain-derived neurotrophic factor (BDNF), retinoic acid, peptides able to facilitate active conformation of activators, agents able to stimulate
15   expression of activator genes; the administration alone or in combination of agents able to decrease the activity and/or concentration of inhibitors of plasmin or plasmin activators, such as T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione), analapril, peptides able to block active conformation of inhibitors, agents able to inhibit expression of inhibitor genes. Plasmin-like activity is defined as the ability to cleave
20   a plasmin substrate such as S-2251.

The present invention relates to methods of causing an increase in plasmin or plasmin-like activity in a cell. Such methods include contacting a cell alone or in combination with compositions having plasmin or plasmin-like activity, such as plasmin, plasminogen, chimeric plasminogen; the administration of activators of plasmin, such as
25   tissue-type plasminogen activator, urokinase-type plasminogen activator, α-enolase, staphylokinase, aspirin; the administration of agents able to enhance activator activity or concentration, such as sulodexide, recombinant brain-derived neurotrophic factor (BDNF), retinoic acid, peptides able to facilitate active conformation of activators, agents able to stimulate expression of activator genes; the administration alone or in combination of
30   agents able to decrease the activity and/or concentration of inhibitors of plasmin or plasmin activators, such as T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione), analapril, peptides able to block active conformation of inhibitors, agents able to inhibit expression of inhibitor genes. Plasmin-like activity is defined as the ability to cleave a plasmin substrate such as S-2251.
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The present invention further relates to compositions useful for increasing the plasmin or plasmin-like activity in a cell or in a subject's brain. Such compositions may include, alone or in combination, plasmin, plasminogen, chimeric plasminogen, tissue-type plasminogen activator, urokinase-type plasminogen activator, staphylokinase, aspirin, sulodexide, recombinant brain-derived neurotrophic factor (BDNF), retinoic acid, peptides

5   able to facilitate active conformation of activators, agents able to stimulate expression of plasminogen or plasminogen activator genes, T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione), analapril, peptides able to block active conformation of inhibitors, and agents able to inhibit expression of inhibitor genes, agents able to enhance expression of plasminogen or plasminogen activator genes.

10   The present invention also relates to methods of identifying compositions useful for treating Alzheimer's disease in a subject. Such methods can include contacting a test compound with a cell line expressing APP and able to process APP to both the p3 and Aβ forms and assaying the production of p3 and Aβ. Compounds capable of increasing the ratio of p3 to Aβ produced by the cells relative to the ratio of p3 to Aβ produced by non-

15   treated control cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention. Preferably, the compound causes a decrease in the amount of Aβ produced. In another embodiment, a cell-free assay is utilized. In the cell-free assay, a substrate, e.g., APP, a fragment of APP comprising the α-secretase site or a peptide comprising the α-secretase site, is contacted with a solution

20   comprising a protease, e.g., plasmin, a fragment of plasmin comprising the active site of plasmin, or a peptide comprising the active site of plasmin, in the presence or absence of a test compound. Compounds capable of increasing cleavage of the substrate by the protease at the α-secretase site relative to the cleavage by the protease in the absence of the test compound are identified as potential therapeutic agents useful for the treatment of

25   Alzheimer's disease according to the invention.

Such methods can also include contacting a test compound with a cell line expressing APP and able to process APP to both the p3 and Aβ forms, or a cell line expressing p3 or Aβ directly, and assaying the degradation of p3 or Aβ. Compounds capable of increasing the degradation of p3 or Aβ as compared to the degradation in control

30   cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention. Preferably, the compound causes a decrease in the amount of Aβ produced. In another embodiment, a cell-free assay is utilized. In the cell-free assay, a substrate, e.g., Aβ, p3, a fragment of Aβ or p3 comprising the plasmin degradation site or a peptide comprising the plasmin degradation site, is contacted with a

35   solution comprising a protease, e.g., plasmin, a fragment of plasmin comprising the active site of plasmin, or a peptide comprising the active site of plasmin, in the presence or

absence of a test compound. Compounds capable of increasing cleavage of the substrate relative to the cleavage of the substrate in the absence of the test compound are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention.

Diagnostic and screening methods for both familial and sporadic
5   Alzheimer's disease are also provided.

4.   BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict the proteins present in the raft fraction of stage 5 neurons as detected by 2D electrophoresis. Figure 1A represents all proteins in the raft
10 fraction, while Figure 1B represents only those protein still present after removal of sphingomyelin or cholesterol.

Figures 2A and 2B depict the proteins present in the raft fraction of treated and untreated stage 5 and stage 4 neurons as detected by 2D electrophoresis.
Figure 2A shows 2D gel autoradiography of the raft fractions from: labeled stage 5
15 hippocampal neurons (Stage 5); labeled stage 5 neurons treated with Fumonisin B, an inhibitor of sphingolipid synthesis (Stage 5 + FB); labeled stage 5 neurons treated with methyl-b-cyclodextrin that extracts cholesterol from the cell membranes (Stage 5 +CD).
Figure 2B shows 2D gel autoradiography of the raft fractions from labeled stage 4 hippocampal neurons (Stage 4) and labeled stage 4 hippocampal neurons treated with
20 ceramide (Stage 4 + Ceramide).

Figure 3A depicts the localization of plasmin and α-enolase on the surface of hippocampal neurons as detected immunofluorescence staining. Figure 3B shows the Western blot analysis of the raft fraction of stage 5 hippocampal neurons using a polyclonal antibody against plasminogen. Molecular weight markers in kD are depicted on the left.
25 Figure 4 depicts a Western Blot analysis of all the fractions of an optiprep gradient used to obtain the rafts (as indicated in materials and methods). Molecular weight markers in kD are shown on the left.

Figure 5A depicts representative autoradiographs of α and β cleavage products of APP immunoprecipitated from treated and non-treated stage 5 hippocampal cell
30 neurons. The cells were treated with either plasmin, plasminogen, plasminogen activator inhibitor, or lysine. Figure 5B summarizes the results obtained from two or three different experiments performed in each particular condition.

Figure 6A depicts an autoradiograph of α and β cleavage products of APP immunoprecipitated from treated and non-treated stage 5 hippocampal cell neurons. The
35 cells were incubated in the presence and absence of plasmin. Figure 6B depicts the

quantification of the intensities of the bands depicted in Figure 6A expressed relative to the control values.

Figure 7A depicts an autoradiograph of the α cleavage products of APP immunoprecipitated from the media surrounding treated and non-treated stage 5 hippocampal cell neurons. The cells were incubated in the presence and absence of tPA.

5   Figure 7B depicts an autoradiograph of the level of expression of APP in both the treated and control cultures.

Figure 8A depicts quantification (by scanning densitometry) of an autoradiograph of the α and β cleavage products of APP immunoprecipitated from the media surrounding treated and non-treated HEK cells expressing the human isoform of

10 APP695. The cells were incubated in the presence and absence of tPA. Figure 8B depicts an autoradiograph of the level of secreted Aβ and p3 from HEK cells expressing the human isoform of APP695 incubated in the presence or absence of several concentrations of plasmin.

15 **5.   DETAILED DESCRIPTION OF THE INVENTION**

Since patients with Alzheimer's disease of genetic origin show an increased activity of Aβ producing secretases or higher susceptibility of the Aβ cleavage sites, a commonly proposed mechanisms for Alzheimer's disease is that the disease is caused by a "gain of function" defect, i.e., increased cleavage at the β and γ-secretase sites. An

20 alternative view would be however, that non-genetic Alzheimer's disease appears after a decreased activity of the mechanisms responsible for cleavage at the α-secretase site, which cleavage precludes the formation of Aβ. It has been discovered that plasmin, or a plasmin-like activity, is involved in the processing of Aβ. Blocking of plasmin activity results in a reduction of APP cleavage at the α-secretase site and an increase in cleavage of APP at the

25 β-secretase site. In addition, diminution in Aβ clearance is also observed. It has also been discovered that increasing plasmin or plasmin-like activity in a cell causes the degradation of Aβ. It is an object of the invention to identify agents capable of increasing cleavage of APP at the α-secretase site, degradation of Aβ, and clearance of Aβ for use in the treatment of Alzheimer's disease. Cleavage at the α-secretase site is known to preclude cleavage at

30 the β-secretase site. Since the latter is necessary for formation of Aβ, enhancement of the former is expected to reduce the formation of Aβ, and, consequently, to reduce or eliminate the formation of Aβ plaques. Also, plasmin or plasmin-like activity is able to degrade Aβ. Thus, it is an object of the present invention to provide compositions useful for increasing the cleavage of APP at the α-secretase site or for increasing the degradation of Aβ by

35 enhancing the activity of plasmin, and the use of such compositions for the treatment of Alzheimer's disease.

5.1.   TREATMENT OF ALZHEIMER'S DISEASE

The invention provides a method of treating or preventing Alzheimer's disease (AD) with a composition capable of enhancing cleavage of APP at the α-secretase site by increasing plasmin or plasmin-like activity. The invention also provides a method of treating or preventing Alzheimer's disease (AD) with a composition capable of enhancing
5   degradation of Aβ by increasing plasmin or plasmin-like activity.

The invention also provides a method of enhancing plasmin or plasmin-like activity in a cell by contacting the cell with a composition capable of increasing plasmin or plasmin-like activity. The invention also provides a method of enhancing cleavage of APP at the α-secretase site in a cell by contacting the cell with a composition capable of
10   increasing plasmin or plasmin-like activity. The invention also provides a method of enhancing degradation of Aβ in a cell by contacting the cell with a composition capable of increasing plasmin or plasmin-like activity.

Examples of such compositions include compositions comprising plasmin or plasmin-like activity, such as plasmin, plasminogen, chimeric plasminogen; compositions
15   comprising activators of plasmin, such as tissue-type plasminogen activator, urokinase-type plasminogen activator, staphylokinase, aspirin; compositions comprising agents able to enhance activator activity and/or concentration, such as sulodexide, recombinant brain-derived neurotrophic factor (BDNF), retinoic acid; compositions comprising peptides able to facilitate active conformation of activators; compositions comprising agents able to
20   stimulate expression of plasminogen or plasminogen activator genes; compositions comprising agents able to decrease the activity and/or concentration of inhibitors of plasmin or plasmin activators, such as T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione), analapril, compositions comprising peptides able to block active conformation of inhibitors, and compositions comprising agents able to inhibit
25   expression of inhibitor genes, and compositions comprising any combination of one or more of the foregoing.

In a particular embodiment, the composition comprises one of the above recited agents capable of enhancing plasmin or plasmin-like activity in combination with another agent capable of enhancing plasmin or plasmin-like activity. In a preferred
30   embodiment, the composition of the invention comprises one or more of the agents recited in the preceding paragraph. While each combination of two or more of the agents recited in the preceding paragraph will not be mechanically recited herein, any combination of two or more of the agents recited in the preceding paragraph is specifically and explicitly contemplated for use in the invention.
35   The compositions of the invention may be administered in any appropriate amount in any suitable galenic formulation and following any regime of administration.

The compositions and methods of the invention are useful for the treatment of Alzheimer's disease, the prevention of the onset of the symptoms and signs of Alzheimer's disease, or for the delay of the symptoms and signs in the evolution of the disease. By way of example, but not limitation, methods and compositions of the invention can be used for: 1) patients diagnosed with AD at any clinical stage of the disease, 2) the

5   prevention of AD in patients with early or prodromal symptoms or signs, and 3) the delay of the onset or evolution or aggravation of the symptoms and signs of AD. The methods of the invention will be, for example, useful for the treatment of AD, including the improvement or alleviation of any symptoms and signs of AD, the improvement of any pathological or laboratory findings of AD, the delay of the evolution of AD, the delay of onset of any AD

10 symptoms and signs, as well as the prevention of occurrence of AD, and the prevention of the onset of any of the symptoms and signs of AD.

The actual administered amount is to be decided by the supervising physician and may depend on multiple factors, such as, the age, condition, file history, etc., of the patient in question. Treatment may be monitored by, for example, measuring the

15 plasmin activity present in the subjects cerebrospinal fluid (CSF), blood or plasma. Kits for measuring plasmin activity are commercially available from, for example, American Diagnostica Inc (Greenwich, CT) and American Bioproducts Company/Diagnostica Stago (Parsippany, New Jersey). Treatment may also be monitored by measuring levels of phosphorylated tau protein or Aβ. Phosphorylated tau levels in CSF are high, while Aβ

20 levels in CSF are low in Alzheimer's disease patients (Ishiguro et al., 1999, Neurosci. Lett 270; 91-94 and Andreasen et al., 1999, Arch. Neurol. 56:673-680). The detection of decreasing phosphorylated tau levels or increasing Aβ in the CSF of a patient is indicative of a successful treatment.

The dose can be determined by a physician upon conducting routine

25 experiments. Prior to administration to humans, the efficacy is preferably shown in animal models. Any animal model for Alzheimer's disease known in the art can be used.

The subject, or patient, to be treated using the methods of the invention is an animal, e.g., a mammal, and is preferably human, and can be a fetus, child, or adult.

In a particular embodiment, sulodexide is administered to a subject to treat or

30 prevent Alzheimer's disease. Sulodexide is a fibrinolytic agent that acts by releasing cellular tissue plasminogen activator. It is currently in use for medical purposes (anti thrombosis, diabetic nephropathy, etc). Sulodexide can be administered orally, it has a longer half-life than plasminogen, and has a reduced effect on global coagulation and bleeding parameters (Harenberg, 1998, Med. Res. Rev. 18:1-20).

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Ampotherin may also be used in the compositions and methods according to the invention. Ampotherin increases the activity of plasminogen and the amount of surface-bound plasmin (Parkkinen, 1993, *J. Biol. Chem.* 268:19726-19738).

Recombinant tPA, or tPA analogs, can also be used in the methods and compositions of the invention (Collen *et al.*, U.S. Pat. No. 4,752,603 issued Jun. 21, 1988

5   Anderson *et al.*, U.S. Pat. No. 5,840,564 issued November 24, 1998). Furthermore, the use of chimeric plasminogen activators (*i.e.*, a protein comprising at least a biologically active portion of plasminogen activator fused via a peptide bond to a different protein) is also contemplated. An example of such a chimeric plasminogen activator is K2tu-PA (Asselbergs, *et al.*, 1995, *J. Biotechnol.* 42(3):221-233). The use of other plasminogen

10 activators, such as uPA, α-enolase and γ-enolase, is also contemplated.

The use of saruplase or analogs thereof in the methods and compositions of the invention is also contemplated. Saruplase is a recombinant urokinase-type plasminogen activator (White, 1998, *J Am Coll Cardiol.* 31:487-496).

Plasminogen, streptokinase and urokinases and analogs thereof can also be

15 used in the methods and compositions of the invention. For example, lumbrokinase, which contains plasminogen and plasminogen activator and is currently used for the prevention and treatment of ischemic cerebrovascular disease, may be used according to the invention. It may be given orally in a capsule form. In addition, fragments of plasminogen, streptokinase and urokinases that comprise functional protease domains may also be used.

20 An example of such fragment is described in Burck, *et al.*, 1990, *J. Biol. Chem.* 265:5170-5177, which discloses a fragment of tPA comprising the second kringle and protease domains.

The use of agents capable of stimulating the expression and/or release of plasminogen or plasminogen activators is also contemplated (*J. Biol. Chem.* 265:6104-

25 6111, 1990; *Mol. Cell Biol.* 11:3139-3147, 1991). For example, recombinant brain-derived neurotrophic factor (BDNF), transforming growth factor-β (TGF-β) and retinoic acid are able to increase expression of tPA or uPA (Fiumelli *et al.*, 1999, *Eur. J. Neurosci.* 11:1639-1646; Tran *et al.*, 1999, *Stroke* 30:1671-1677; Lansink *et al.*, 1996, *Blood* 88:531-541). It is also possible to increase the expression of the transcription factor Ets-1, which regulates the

30 expression of uPA (Kitange *et al.*, 1999, *Lab Invest.* 79:407-416). In a particular embodiment, recombinant BDNF is not used in the methods and compositions of the invention. In a further embodiment, TGF-β is not used in the methods and compositions of the invention.

Staphylokinase can also be used in the methods and compositions of the

35 invention. Staphylokinase is a 15 kD bacterial protein that forms a complex with plasmin

which in turn activates other plasminogen molecules by converting them into plasmin (Schlott et al., 1997, J. Biol. Chem. 272:6067-6072).

The use of aspirin in the compositions and methods of the invention is also contemplated. Aspirin has been reported to stimulate plasmin activity (Milwidskyet al., 1991, Thrombo. Haemost. 65:389-393).

5   Reduction of the activity and/or levels of plasminogen activator inhibitors is also contemplated. For example, plasminogen activator inhibitor 1 (PAI-1) is a serpin that has a key role in fibrin degradation through inhibition of plasminogen activation. Inhibition of PAI-1 can be effected by, for example, the specific inhibitor T-686, (3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione. Oral administration in

10   mice prevents death by hypercoagulation (Vinogradsky et al., 1997, Thromb. Res. 85:305-314). Administration of enalapril also inhibits PAI-1. Sakata et al., 1999, Am. Heart J. 137:1094-1099. The use of peptides designed to block the active conformation of PAI-1 or PAI-2 is also contemplated. Sharp, 1999, Struct. Fold. Res. 7:111-118; Harrop, 1999, Struct. Fold. Res. 7:43-54). Agents capable of reducing of the expression of the PAI-1

15   gene, for example by inhibiting the binding of the promoter element B box that binds the helicase-like transcription factor (HLTF), may also be used in the compositions and methods of the invention (Ding et al., 1999, J. Biol. Chem. 274:19573-19580). The use of agents capable of inhibiting the activity of transforming growth factor (TGF)-β1 on the activation of the serpin pathway, which pathway blocks plasminogen activation, is also

20   contemplated. An example of such an agent is a TGF-β neutralizing antibody (Tran et al., 1999, Stroke 30:1671-1678).

In specific embodiments, subjects may be treated with a composition capable of increasing plasmin or plasmin-like activity in the subject, with the proviso that the composition does not contain one or more of the following: plasmin, plasminogen, chimeric

25   plasminogen; activators of plasmin, such as tissue-type plasminogen activator, urokinase-type plasminogen activator, α-enolase, staphylokinase, aspirin; agents able to enhance activator activity or concentration, such as sulodexide, recombinant brain-derived neurotrophic factor (BDNF), retinoic acid, peptides able to facilitate active conformation of activators, agents able to stimulate expression of activator genes; agents able to decrease the

30   activity and/or concentration of inhibitors of plasmin or plasmin activators, such as T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione), analapril, peptides able to block active conformation of inhibitors, and agents able to inhibit expression of inhibitor genes. In a specific embodiment, the composition of the invention or the composition used in a method of the invention does not contain aspirin alone, i.e.,

35   aspirin in the absence of another composition capable of increasing plasmin or plasmin-like activity.

In a specific embodiment of the present invention, a nucleic acid containing a portion of a gene coding for an inhibitor of plasmin activity, such as PAI-1 or PAI-2, in which inhibitor sequences flank (are both 5' and 3' to) a different gene sequence, is used as a plasmin inhibitor antagonist, or to promote plasmin inhibitor inactivation by homologous recombination (see also, Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342: 435-438). The nucleic acid sequences of such inhibitors are known to those of skill in the art, and may be found, for example, in a public database such as Genbank. The sequence for PAI-1 has Genbank accession number M16006 (Ginsburg, *et al.*, 1986, *J. Clin. Invest.* 78(6):1673-1680). The sequence for PAI-2 has Genbank accession number M18082 (Schleuning, *et al.*, 1987, *Mol. Cell. Biol.* 7(12):4564-4567). See section 5.2 herein for exemplary methods of formulating and administering such nucleic acids.

A more specific embodiment of the present invention is directed to a method of reducing plasmin inhibitor expression or activity by targeting mRNAs that express the plasmin inhibitor protein. RNA therapeutics currently fall within three classes, antisense species, ribozymes, or RNA aptamers (Good *et al.*, 1997, *Gene Therapy* 4:45-54).

Antisense oligonucleotides have been the most widely used. By way of example, but not limitation, antisense oligonucleotide methodology to reduce plasmin inhibitor expression is presented below. Ribozyme therapy involves the administration, induced expression, etc. of small RNA molecules with enzymatic ability to cleave, bind, or otherwise inactivate specific RNAs, to reduce or eliminate expression of particular proteins (Grassi and Marini, 1996, *Annals of Medicine* 28:499-510; Gibson, 1996, *Cancer and Metastasis Reviews* 15:287-299). At present, the design of "hairpin" and "hammerhead" RNA ribozymes is necessary to specifically target a particular mRNA such as that for PAI-1 or PAI-2. RNA aptamers are specific RNA ligand proteins, such as for Tat and Rev RNA (Good *et al.*, 1997, *Gene Therapy* 4:45-54) that can specifically inhibit their translation.

In another embodiment, the activity or levels of a plasmin inhibitor are reduced by administration of an antibody that immunospecifically binds to a plasmin inhibitor, or a fragment or a derivative of the antibody containing the binding domain thereof.

In a specific embodiment, a chimeric antibody is used. Techniques have been developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, *Proc. Natl. Acad. Sci.* 81:851-855; Neuberger *et al.*, 1984, *Nature* 312:604-608; Takeda *et al.*, 1985, *Nature* 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region

derived from a murine mAb and a human immunoglobulin constant region, e.g., humanized antibodies.

In a preferred embodiment, a humanized antibody is used, more preferably an antibody having a variable domain in which the framework regions are from a human antibody and the complementarity determining regions are from an antibody of a non-

5 human animal, preferably a mouse (see International Patent Application No. PCT/GB8500392 by Neuberger et al. and Celltech Limited).

Complementarity determining region (CDR) grafting is another method of humanizing antibodies. It involves reshaping murine antibodies in order to transfer full antigen specificity and binding affinity to a human framework (Winter et al. U.S. Patent

10 No. 5,225,539). CDR-grafted antibodies have been successfully constructed against various antigens, for example, antibodies against IL-2 receptor as described in Queen et al., 1989 (Proc. Natl. Acad. Sci. USA 86:10029); antibodies against cell surface receptors-CAMPATH as described in Riechmann et al. (1988, Nature 332:323; antibodies against hepatitis B in Cole et al. (1991, Proc. Natl. Acad. Sci. USA 88:2869); as well as against

15 viral antigens-respiratory syncitial virus in Tempest et al. (1991, Bio-Technology 9:267). CDR-grafted antibodies are generated in which the CDRs of the murine monoclonal antibody are grafted into a human antibody. Following grafting, most antibodies benefit from additional amino acid changes in the framework region to maintain affinity, presumably because framework residues are necessary to maintain CDR conformation, and

20 some framework residues have been demonstrated to be part of the antigen binding site. However, in order to preserve the framework region so as not to introduce any antigenic site, the sequence is compared with established germline sequences followed by computer modeling.

In other embodiments, fusion proteins of modified immunoglobulins(or

25 functionally active fragments thereof) are used. For example, the modified immunoglobulin may be fused via a covalent bond (e.g., a peptide bond), at either the N-terminus or the C-terminus to an amino acid sequence of another protein (or portion thereof, preferably an at least 10, 20 or 50 amino acid portion of the protein) that is not the modified immunoglobulin. Preferably the modified immunoglobulin, or fragment thereof, is

30 covalently linked to the other protein at the N-terminus of the constant domain. In preferred embodiments, fusion proteins are used in which the modified immunoglobulin is covalently linked to IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, γ-interferon, or MHC derived peptide.

The modified immunoglobulins useful in the methods of the invention include analogs and derivatives that are either modified, i.e., by the covalent attachment of

35 any type of molecule as long as such covalent attachment does not prevent the modified immunoglobulin from generating an anti-idiotypic response. For example, but not by way

of limitation, the derivatives and analogs of the modified immunoglobulins include those that have been further modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to

5 specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the analog or derivative may contain one or more non-classical amino acids.

The subject to be treated by the methods and compositions of the invention is preferably a mammal, most preferably a human, but can also be a non-human animal

10 including but not limited to cows, horses, sheep, pigs, fowl (*e.g.*, chickens), goats, cats, dogs, hamsters, mice and rats.

Generally, administration of products of species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, a human plasmin inhibitor protein, or derivative, homolog or

15 analog thereof; nucleic acids encoding human plasmin inhibitor or a derivative, homolog or analog thereof; or humanized, in the case of antibodies, or other human agents that affect plasmin inhibitor expression or activity, are therapeutically or prophylactically administered in an effective amount to a human patient.

In a specific embodiment of the present invention, a nucleic acid containing

20 a portion of a gene coding for an enhancer of plasmin activity, such as plasminogen, tPA, uPA, or α-enolase is used as a plasmin activity agonist. A more specific embodiment of the present invention is directed to a method of increasing plasmin activity by expressing mRNAs that code for an enhancer of plasmin activity (Good *et al.*, 1997, *Gene Therapy* 4:45-54).

25 Generally, administration of products of species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, a human plasmin activity enhancer protein, or derivative, homolog or analog thereof; nucleic acids encoding a human plasmin activity enhancer or a derivative, homolog or analog thereof; or other human agents that affect plasmin enhancer

30 expression or activity, are therapeutically or prophylactically administered to a human patient.

Preferably, suitable *in vitro* or *in vivo* assays are utilized to determine the effect of a specific therapeutic agent and whether its administration is indicated for treatment of the affected tissue or individual.

35 In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a

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therapeutic agent has a desired effect upon such cell types. The representative cells are preferably rat hippocampal neurons. The cells may also be CHO or COS cells engineered to express a peptide comprising at least a portion of wild type or a mutant APP. Such cells may also be further engineered to express a peptide comprising at least a biologically active portion of plasminogen and/or a plasminogen activator, such as t-PA.

5   Therapeutic agents for use in therapy can be tested in suitable animal model systems prior to testing in humans, including, but not limited to, rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

10   **5.2.   GENE THERAPY**

In a specific embodiment of the present invention, nucleic acids comprising a sequence encoding a plasmin activity enhancer, such as plasmin, plasminogen, tPA, uPA, γ-enolase or α-enolase, or a functional derivative thereof, are administered to enhance plasmin activity by way of gene therapy. Gene therapy refers to therapy performed by the

15   administration of a nucleic acid to a subject. In this embodiment of the present invention, the nucleic acid expresses its encoded protein(s) that mediates a therapeutic effect by modulating plasmin activity. Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

The nucleic acid sequences of the above proteins are known to those of skill

20   in the art, and may be found, for example, in a public database such as Genbank. The sequence for PAI-1 has Genbank accession number M16006 (Ginsburg, *et al.*, 1986, *J. Clin. Invest.* 78(6):1673-1680). The sequence for PAI-2 has Genbank accession number M18082 (Schleuning, *et al.*, 1987, *Mol. Cell. Biol.* 7(12):4564-4567). The sequence for tPA has Genbank accession number M15518 (Harris, *et al.*, 1986, *Mol. Biol. Med.* 3(3):279-292).

25   The sequence for uPA has Genbank accession number M15476 (Holmes, *et al.*, 1985, *Biotechnology* (N.Y.) 3:923-929. The sequence for γ-enolase has Genbank accession number M22349 M27833 (Oliva, *et al.*, 1989, *Gene* 79(2):355-360). The sequence for α-enolase has Genbank accession number M14328 (Giallongo, *et al.*, 1986, *Proc. Natl. Acad. Sci.* 83(18):6741-6745).

30   For general reviews of the methods of gene therapy, see Goldspiel *et al.*, 1993, *Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; and May, 1993, *TIBTECH*

35   11:155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.*, eds., 1993, Current Protocols in Molecular

Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the therapeutic agent comprises a nucleic acid coding for an enhancer of plasmin activity that is part of an expression vector that expresses said enhancer of plasmin activity. In particular, such a nucleic acid has a promoter operably

5   linked to the plasmin activity enhancer coding region, said promoter being inducible or constitutive, and optionally, tissue-specific, preferably brain-specific or neuron specific. In another particular embodiment, a nucleic acid molecule is used in which the coding sequence, and any other desired sequences, are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intra-chromosomal

10   expression of the nucleic acid (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra *et al.*, 1989, *Nature* 342:435-438). In another preferred aspect, the nucleic acid is an antisense nucleic acid that inhibits the expression of an inhibitor of plasmin activity.

Plasmin inhibitor function may be inhibited by use of antisense nucleic

15   acids. The present invention provides the therapeutic or prophylactic use of nucleic acids of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 200 oligonucleotides), that are antisense to a gene or cDNA encoding a plasmin inhibitor, or portions thereof. A plasmin inhibitor "antisense" nucleic acid as used herein refers to a nucleic acid capable of hybridizing to a portion of a plasmin inhibitor nucleic acid

20   (preferably mRNA) by virtue of some sequence complementarity. The antisense nucleic acid may be complementary to a coding and/or noncoding region of a plasmin inhibitor mRNA. In specific aspects, the oligonucleotide is at least 6 nucleotides, at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified

25   versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at any position (examples of such modifications can be found in: Bailey, Ullmann's Encyclopedia of Industrial Chemistry (1998), 6th ed. Wiley and Sons). Such antisense nucleic acids have utility in inhibiting plasmin inhibitor function or activity, and can be used in the treatment or prevention of Alzheimer's disease.

30   The plasmin inhibitor antisense nucleic acids can be directly administered to a cell, or can be produced intracellularly by transcription of exogenous, introduced sequences. Alternatively, plasmin inhibitor antisense nucleic acids are produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion

35   thereof is transcribed, producing an antisense nuclcio acid (RNA) of the invention. Such a vector can remain episomal or become chromosomally integrated, as long as it can be

transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a plasmin inhibitor gene, preferably a human plasmin inhibitor gene. However, absolute complementarity, although

5   preferred, is not required.

The amount of plasmin inhibitor antisense nucleic acid that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity *in vitro*, and then in useful

10   animal model systems prior to testing and use in humans.

In a specific embodiment, pharmaceutical compositions comprising plasmin inhibitor antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the plasmin inhibitor antisense nucleic acids.

15   In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable central nervous system cell types (Leonetti *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87:2448-2451; Renneisen *et al.*, 1990, *J. Biol. Chem.* 265:16337-16342).

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect,

20   in which case, cells are first transformed with the nucleic acid in vitro, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of

25   numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors, or through use of

30   transfecting agents, by encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide that is known to enter the nucleus, or by administering it in linkage to a ligand subject to receptor-mediated endocytosis that can be used to target cell types specifically expressing the receptors (*e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432), etc. In another embodiment, a nucleic acid-ligand complex

35   can be formed in which the ligand comprises a fusogenic viral peptide that disrupts endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another

embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, e.g., International Patent Publications WO 92/06180; WO 92/22635; WO 92/20316; WO 93/14188; and WO 93/20221. Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA*

5   86:8932-8935; Zijlstra *et al.*, 1989, *Nature* 342:435-438).

In a specific embodiment, a viral vector that contains the plasmin activity enhacer encoding nucleic acid is used. In another specific embodiment, a viral vector that contains an inhibitor of plasmin activity antisense nucleic acid is used. For example, a retroviral vector can be used (Miller *et al.*, 1993, *Meth. Enzymol.* 217:581-599). These

10   retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The coding or antisense nucleic acids to be used in gene therapy is/are cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen *et al.*, 1994, *Biotherapy* 6:291-302, which describes the use of a retroviral

15   vector to deliver the mdr1 gene to hematopoetic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are Clowes *et al.*, 1994, *J. Clin. Invest.* 93:644-651; Kiem *et al.*, 1994, *Blood* 83:1467-1473; Salmons and Gunzberg, 1993, *Human Gene Therapy* 4:129-141; and Grossman and Wilson, 1993, *Curr. Opin. in Genetics and Devel.* 3:110-114.

20   Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are the liver, the central nervous system, endothelial cells (such as prostate cells) and muscle. Adenoviruses have the advantage of

25   being capable of infecting non-dividing cells (Kozarsky and Wilson, 1993, *Current Opinion in Genetics and Development* 3:499-503, discuss adenovirus-based gene therapy). The use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys has been demonstrated by Bout *et al.*, 1994, *Human Gene Therapy* 5:3-10. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.*, 1991, *Science*

30   252:431-434; Rosenfeld *et al.*, 1992, *Cell* 68:143-155; and Mastrangeli *et al.*, 1993, *J. Clin. Invest.* 91:225-234.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh *et al.*, 1993, *Proc. Soc. Exp. Biol. Med.* 204:289-300).

Another approach to gene therapy involves transferring a gene into cells in

35   tissue culture by methods such as electroporation, lipofection, calcium phosphate-mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a

selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene from these that have not. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried

5 out by any method known in the art including, but not limited to, transfection by electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, e.g., Loeffler and Behr, 1993, *Meth.*

10 *Enzymol.* 217:599-618; Cohen *et al.*, 1993, *Meth. Enzymol.* 217:618-644; Cline, 1985, *Pharmac. Ther.* 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably, is heritable and

15 expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, cells are injected, *e.g.*, into the brain. Recombinant blood cells (*e.g.*, hematopoetic stem or progenitor cells) are preferably administered intravenously. The number of cells envisioned for use depends on the desired

20 effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, neuronal cells, hippocampal cells, fibroblasts, muscle cells, hepatocytes, blood cells such as T lymphocytes, B lymphocytes,

25 monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, and granulocytes, various stem or progenitor cells, in particular hematopoetic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

30 In one embodiment in which recombinant cells are used in gene therapy, a plasmin activity enhancer encoding nucleic acid is introduced into the cells such that the gene is expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained in

35 vitro can potentially be used in accordance with this embodiment of the present invention. Such stem cells include but are not limited to hematopoetic stem cells (HSCs), stem cells of

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epithelial tissues such as the skin and the lining of the gut, embryonic heart muscle cells, liver stem cells (International Patent Publication WO 94/08598), and neural stem cells (Stemple and Anderson, 1992, *Cell* 71:973-985).

Epithelial stem cells (ESCs), or keratinocytes, can be obtained from tissues such as the skin and the lining of the gut by known procedures (Rheinwald, 1980, *Meth.*
5   *Cell Biol.* 2A:229). In stratified epithelial tissue such as the skin, renewal occurs by mitosis of stem cells within the germinal layer, the layer closest to the basal lamina. Similarly, stem cells within the lining of the gut provide for a rapid renewal rate of this tissue. ESCs or keratinocytes obtained from the skin or lining of the gut of a patient or donor can be grown in tissue culture (Rheinwald, 1980, *Meth. Cell Bio.* 2A:229; Pittelkow and Scott, 1986,
10 *Mayo Clinic Proc.* 61:771). If the ESCs are provided by a donor, a method for suppression of host versus graft reactivity (*e.g.*, irradiation, or drug or antibody administration to promote moderate immunosuppression) can also be used.

With respect to hematopoetic stem cells (HSCs), any technique that provides for the isolation, propagation, and maintenance in vitro of HSCs can be used in this
15 embodiment of the invention. Techniques by which this may be accomplished include (a) the isolation and establishment of HSC cultures from bone marrow cells isolated from the future host, or a donor, or (b) the use of previously established long-term HSC cultures, which may be allogeneic or xenogeneic. Non-autologous HSCs are used preferably in conjunction with a method of suppressing transplantation immune reactions between the
20 future host and patient. In a particular embodiment of the present invention, human bone marrow cells can be obtained from the posterior iliac crest by needle aspiration (see, *e.g.*, Kodo *et al.*, 1984, *J. Clin. Invest.* 73:1377-1384). In a preferred embodiment of the present invention, the HSCs can be made highly enriched or in substantially pure form. This enrichment can be accomplished before, during, or after long-term culturing, and can be
25 done by any technique known in the art. Long-term cultures of bone marrow cells can be established and maintained by using, for example, modified Dexter cell culture techniques (Dexter *et al.*, 1977, *J. Cell Physiol.* 91:335) or Witlock-Witte culture techniques (Witlock and Witte, 1982, *Proc. Natl. Acad. Sci. USA* 79:3608-3612).

In a specific embodiment, the nucleic acid to be introduced for purposes of
30 gene therapy comprises an inducible promoter operably linked to the coding or antisense region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

5.3. PHARMACEUTICAL COMPOSITIONS
35
The pharmaceutical compositions according to the present invention preferably comprise one or more pharmaceutically acceptable carriers and the active

constituents. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

In a preferred embodiment, the active ingredient of the pharmaceutical composition according to the present invention is purified. In specific embodiments, the active ingredient is a protein, and is purified to be 50%, 75%, 90%, 95% or 99% pure

5 protein by weight.

It will be appreciated that the amounts of the active constituents required for treatment or prevention of Alzheimer's disease will vary according to the route of administration, the condition, age, and file history of the subject, the galenic formulation of the pharmaceutical composition, etc.

10 The actually administered amounts of active constituents may be decided by a supervising physician.

Therapeutic formulations include those suitable for parenteral (including intramuscular and intravenous), oral rectal or intradermal administration, although oral administration is the preferred route. Thus, the pharmaceutical composition may be

15 formulated as tablets, pills, syrups, capsules, suppositories, formulations for transdermal application, powders, especially lyophilized powders for reconstitution with a carrier for intravenous administration, etc..

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. The carriers in the pharmaceutical composition may

20 comprise a binder, such as microcrystalline cellulose, polyvinylpyrrolidone (polyvidone or povidone), gum tragacanth, gelatine, starch, lactose or lactose monohydrate; a disintegrating agent, such as alginic acid, maize starch and the like; a lubricant or surfactant, such as magnesium stearate, or sodium lauryl sulphate; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; and/or a flavoring agent, such as

25 peppermint, methyl salicylate, or orange flavoring.

Therapeutic formulations suitable for oral administration, e.g., tablets and pills, may be obtained by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing one or more active constituents in a suitable apparatus into tablets having a suitable size. Prior to the mixing,

30 the one or more active constituents may be mixed with a binder, a lubricant, an inert diluent and/or a disintegrating agent, a diluent, a lubricant and/or a surfactant.

In a preferred embodiment, one or more active constituents are mixed with a binder, such as microcrystalline cellulose until a homogeneous mixture is obtained. Subsequently, another binder, such as polyvidone, is transferred to the mixture under

35 stirring. This mixture is passed through granulating sieves and dried by desiccation before compression into tablets in a standard compressing apparatus.

A tablet may be coated or uncoated. An uncoated tablet may be scored. A coated tablet may be coated with sugar, shellac, film or other enteric coating agents.

Therapeutic formulations suitable for parenteral administration include sterile solutions or suspensions of the active constituents. An aqueous or oily carrier may be used. Such pharmaceutical carriers can be sterile liquids, such as water and oils,

5   including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Formulations for parenteral administration also include a lyophilized powder comprising one or more active constituents that is to be reconstituted by dissolving in a pharmaceutically acceptable carrier that dissolves said one or more active constituents, e.g., an aqueous solution of carboxymethylcellulose and lauryl

10   sulfate.

When the pharmaceutical composition is a capsule, it may contain a liquid carrier, such as a fatty oil, e.g., cacao butter.

Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc,

15   sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

In yet another embodiment, the therapeutic compound can be delivered in a

20   controlled release system. In one embodiment, a pump may be used (see Langer, supra;
. Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug.

25   Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction

30   of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In one embodiment of the pharmaceutical composition according to the

35   invention, two or more active constituents are comprised as separate entities. The two entities may be administered simultaneously or sequentially.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of

5   manufacture, use or sale for human administration.

In a preferred embodiment, native or recombinant t-PA is administered by intravenous (i.v.) injection. The invention also provides a pharmaceutical pack or kit comprising one or more sterile syringes and containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with

10 such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5.4. SCREENING FOR POTENTIAL ALZHEIMER'S DISEASE THERAPEUTICS

15

The present invention also relates to methods of identifying compositions useful for the treatment of prevention of Alzheimer's disease. Such methods can include co-culturing a cell that is transformed with and expresses a nucleic acid encoding plasminogen and a cell that is transformed with and expresses a substrate, e.g., an amyloid

20 precursor protein, a mutant form thereof having a mutation associated with familial Alzheimer's disease, a fragment thereof comprising a protease cleavage site, or a peptide comprising a protease cleavage site, in the presence of one or more candidate molecules and then determining the rate or amount of β-amyloid production that occurs, wherein an increase in said rate or amount relative to the rate or amount in the absence of the candidate

25 molecules indicates that the candidate molecules are a potential therapeutic for Alzheimer's disease. In another embodiment, the cell used in the screen is transformed with and expresses a nucleic acid encoding plasminogen as well as a nucleic acid encoding and expressing a substrate, e.g., an amyloid precursor protein, a mutant form thereof having a mutation associated with familial Alzheimer's disease, a fragment thereof comprising a

30 protease cleavage site, or a peptide comprising a protease cleavage site. The protease cleavage site may be the α-secretase site, the β-secretase site, or the plasmin degradation site. "Co-culturing" includes culturing two different cells, one expressing the amyloid protein, the other expressing the plasminogen, or culturing a single cell expressing both the amyloid protein and the plasminogen. Additional component, such as activators of

35 plasminogen, e.g., t-PA, and/or inhibitors of plasminogen activity, e.g., PAI-1 or PAI-2, may also be added to the co-culture. These additional components may be provided either

as proteins added to the co-culture, or by further co-culturing cells capable of expressing them. As above, "co-culturing" can take the form of either culturing an additional cell expressing the appropriate protein, or culturing a cell expressing more than one of the desired proteins.

5   In an alternative embodiment, the cell expressing amyloid precursor protein, a mutant form thereof having a mutation associated with familial Alzheimer's disease or a fragment thereof comprising a protease cleavage site is incapable of expressing the activity of cleaving amyloid precursor at or near the α-secretase site or degrading Aβ and/or p3, or if such activity is expressed by the cell, the amount of amyloid precursor processing mediated by such activity is insignificant compared to the activity mediated by the

10 plasminogen and/or other proteins expressed by the co-cultured cells. Preferably, the amyloid precursor processing at the α-secretase site mediated by the plasminogen and/or other proteins produced by the co-cultured cells is at least 70%, at least 80%, at least 90% or at least 95% of the total amount of amyloid precursor processing at the α-secretase site taking place in the co-culture. When the activity to be measured is degradation of Aβ or p3,

15 preferably the degradation mediated by the plasminogen and/or other proteins produced by the co-cultured cells is at least 70%, at least 80%, at least 90% or at least 95% of the total amount of degradation taking place in the co-culture.

     The present invention also provides a kit comprising in one or more containers a cell that is transformed with and expresses a nucleic acid encoding

20 plasminogen and a cell that is transformed with and expresses a substrate, e.g., an amyloid precursor protein, a mutant form thereof having a mutation associated with familial Alzheimer's disease, a fragment thereof comprising a protease cleavage site, or a peptide comprising a protease cleavage site. In another embodiment, the cell comprising the kit is transformed with and expresses a nucleic acid encoding plasminogen as well as a nucleic

25 acid encoding and expressing a substrate, e.g., an amyloid precursor protein, a mutant form thereof having a mutation associated with familial Alzheimer's disease, a fragment thereof comprising a protease cleavage site, or a peptide comprising a protease cleavage site. The present invention also provides a kit comprising in one or more containers a cell that is transformed with and expresses a nucleic acid encoding a substrate, e.g., an amyloid

30 precursor protein, a mutant form thereof having a mutation associated with familial Alzheimer's disease, a fragment thereof comprising a protease cleavage site, or a peptide comprising a protease cleavage site. In another embodiment, the kit comprises in one or more containers a cell that naturally expresses a nucleic acid encoding a substrate, e.g., an amyloid precursor protein, a mutant form thereof having a mutation associated with familial

35 Alzheimer's disease, a fragment thereof comprising a protease cleavage site, or a peptide comprising a protease cleavage site.

5.4.1. SCREENING FOR AGENTS CAPABLE OF INCREASING PLASMIN OR PLASMIN ACTIVATOR ACTIVITY

The present invention also relates to methods of identifying compositions useful for increasing plasmin or plasmin-like activity in a subject's brain. Such methods can include contacting a test compound with a cell line expressing plasminogen and assaying plasmin and/or plasmin activator (e.g., tPA, uPA, α-enolase, γ-enolase) activity. While all methods of assaying plasmin activity are contemplated in the present invention, an example of such assay is the detection of cleavage of the plasmin substrate S-2251, as described in United States Patent 5,840,564, issued November 24, 1998. A method for measuring plasmin activator activity has also been described (United States Patent No. 5,175,087; Wiman et al., 1983, Clin Chem Act., 127:279-288). Compounds capable of increasing plasmin or plasmin activator activity produced by the cells relative to that produced by non-treated control cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention. In another embodiment, a cell-free assay is utilized. In the cell-free assay, plasminogen or plasmin is incubated in the presence or absence of a test compound and a known substrate for plasmin. An increase in the amount of cleaved substrate in the presence of the compound relative to the amount cleaved in the absence of the compound indicates that the compound increases plasmin activity. Known activators of plasminogen, such as tPA, and/or known inactivators of plasminogen, such as PAI-1, can also be included in the assay. Compounds capable of increasing plasmin or plasmin activator activity are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention.

Compounds may also be tested for their ability to increase plasmin or plasmin activator activity in the serum, CSF, brain, or other fluid or tissue of animals, including humans. A test compound can be administered to an animal, e.g., a mouse or monkey, followed by the determination of plasmin-activity in the serum, CSF and/or brain of the animal. Compounds able to increase the plasmin or plasmin activator activity present in one or more of the tested samples are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention.

Often it is desirable to first identify potential lead compounds by their ability to bind to a target molecule. Using this approach, one would incubate a target molecule, e.g., plasmin, plasminogen, tPA, uPA, PAI-1, PAI-2, etc., with a series of test compounds and assay for binding of the test compound to the target molecule. Assaying for simple binding, as opposed to activity, allows for rapid throughput screening. Those compounds capable of binding to the target molecule are then assayed for their effects on the activity of the relevant target protein. Such binding assays may be performed with fragments of the target molecule instead of the full length target molecule. Preferably, the fragment

comprises the active site of the target molecule. Binding may be detected by using labeled, e.g., radioactive or fluorescent labels, test compounds and assaying for the association of label with target molecule. To facilitate such an assay, the target molecule may be immobilized by, for example, coating the target molecule on the bottom of a test chamber, such as a well of a 96-well microtiter plate. The target may also be immobilized by binding

5 to an antibody affixed to a test chamber.

In another aspect, binding of plasmin and a substrate, e.g., amyloid precursor protein, a fragment of APP comprising the α-secretase site, a peptide comprising the α-secretase site, Aβ, p3, a fragment of Aβ or p3 comprising the plasmin degradation site, or a peptide comprising the plasmin degradation site may be assayed. Compounds able to

10 enhance the binding of the amyloid protein to the substrate are identified as potential therapeutic agents. Similarly, binding of inhibitors (e.g., PAI-1) to activators (e.g., tPA) may also be assayed. Compounds able to inhibit the binding of an inhibitor to an activator are identified as potential therapeutic agents.

15   **5.4.2. SCREENING FOR AGENTS CAPABLE OF
              INCREASING DESIRABLE PROCESSING OF APP**

The present invention also relates to methods of identifying compositions useful for increasing plasmin or plasmin-like activity in a subject's brain. Such methods can include contacting a test compound with a cell line expressing APP and able to process

20 APP to both the p3 and Aβ forms and assaying the production of p3 and Aβ. Preferably, test compounds have been first assayed for their ability to increase plasmin activity, as described in section 5.4.1. Compounds capable of increasing the ratio of p3 to Aβ produced by the cells relative to the ratio of p3 to Aβ produced by non-treated control cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease

25 according to the invention. Preferably, the compound causes a decrease in the amount of Aβ produced.

In another embodiment, a cell-free assay is utilized. In the cell-free assay, a substrate, e.g., APP, a fragment of APP comprising the α-secretase site or a peptide comprising the α-secretase site, is contacted with a solution comprising a protease, e.g.,

30 plasmin, a fragment of plasmin comprising the active site of plasmin, or a peptide comprising the active site of plasmin, in the presence or absence of a test compound. Other proteins may be added, such as inactive proteases that may be activated by the activity of the first protease. Compounds capable of increasing cleavage of the substrate by the one or more proteases at the α-secretase site relative to the cleavage by the protease in the absence

35 of the test compound are identified as potential therapeutic agents useful for the treatment of

Alzheimer's disease according to the invention. Preferably, the compound causes a decrease in the amount of Aβ produced.

5.4.2.1   SCREENING FOR AGENTS CAPABLE OF INCREASING α CLEAVAGE

5   In one embodiment, the assay comprises incubating a cell line (e.g., CHO, COS) constitutively expressing a fusion protein of human APP tagged with green fluorescent protein (GFP), or other suitable fluorescent protein, at the N-terminal in the presence or absence of a test compound and monitoring the levels of GFP tagged p3 fragment in the medium (α cleavage occurs in the extracellular side of the protein) from

10   treated and untreated cells by ELISA assay using an antibody against GFP. (That is, a solid phase to which anti-GFP antibody is attached is contacted with the culture medium or sample derived therefrom, and fluorescence on the solid phase is measured.) All samples in which there is an increase in p3 production are further analyzed separately by conventional biochemical assays (e.g., by using specific antibodies) to determine the amount of secreted

15   p3. Test compounds capable of increasing production of p3 and/or reducing the production of Aβ produced by the cells relative to that produced by non-treated control cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention. Alternatively, or in addition, changes in production of Aβ can also be determined. Test compounds capable of decreasing production of Aβ produced by

20   the cells relative to that produced by non-treated control cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention.

5.4.3.   SCREENING FOR AGENTS CAPABLE OF INCREASING DEGRADATION OF Aβ

25

The present invention also relates to methods of identifying compositions useful for increasing plasmin or plasmin-like activity in a cell or a subject's brain to cause the degradation of Aβ. Such methods can include contacting a test compound with a cell line expressing APP and able to process APP to both the p3 and Aβ forms and assaying the

30   production of p3 and Aβ. Preferably, test compounds have been first assayed for their ability to increase plasmin activity, as described in section 5.4.1. Compounds capable of increasing the degradation of p3 and/or Aβ produced by the cells relative to the degradation produced by non-treated control cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention.

35   In another embodiment, a cell-free assay is utilized. In the cell-free assay, a substrate, e.g., APP, Aβ, p3, a fragment of APP comprising the plasmin degradation site or

a peptide comprising the plasmin degradation site is contacted with a solution comprising a protease, e.g., plasmin, a fragment of plasmin comprising the active site of plasmin, or a peptide comprising the active site of plasmin, in the presence or absence of a test compound. Other proteins may be added, such as inactive proteases that may be activated by the activity of the first protease. Compounds capable of increasing cleavage of the

5   substrate by the one or more proteases at the plasmin degradation site relative to the cleavage by the protease in the absence of the test compound are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention.

In one embodiment, the assay comprises incubating a cell line (e.g., CHO,

10   COS) constitutively expressing a fusion protein of human APP tagged with green fluorescent protein (GFP), or other suitable fluorescent protein, at the N-terminal in the presence or absence of a test compound and monitoring the levels of GFP tagged Aβ fragment in the medium from treated and untreated cells by ELISA assay using an antibody against GFP. (That is, a solid phase to which anti-GFP antibody is attached is contacted

15   with the culture medium or sample derived therefrom, and fluorescence on the solid phase is measured.) All samples in which there is a decrease in Aβ production are further analyzed separately by conventional biochemical assays (e.g., by using specific antibodies) to determine the amount of secreted Aβ and the amount of degradative products of Aβ. Test compounds capable of decreasing the amount of Aβ and/or increasing the degradation

20   of Aβ produced by the cells relative to that produced by non-treated control cells are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the invention.

In another embodiment, the assay comprises incubating a cell line (e.g., CHO, COS) constitutively expressing the Aβ or p3 fragment of human APP tagged with

25   green fluorescent protein (GFP), or other suitable fluorescent protein, at the N-terminal in the presence or absence of a test compound and monitoring the levels of GFP tagged Aβ or p3 in the medium from treated and untreated cells by ELISA assay using an antibody against GFP. (That is, a solid phase to which anti-GFP antibody is attached is contacted with the culture medium or sample derived therefrom, and fluorescence on the solid phase

30   is measured.) All samples in which there is reduction in Aβ or p3 are further analyzed separately by conventional biochemical assays (e.g., by using specific antibodies) to determine if degradation of Aβ or p3 has occurred. Test compounds capable of decreasing the amount of Aβ or p3 and/or increasing the degradation of Aβ or p3 produced by the cells relative to that produced by non-treated control cells are identified as potential therapeutic

35   agents useful for the treatment of Alzheimer's disease according to the invention.

Cell free assays can also be used. For example, a substrate, *e.g.*, recombinantly produced Aβ or p3 peptide, can be incubated in the presence of a protease, such as plasmin, in the presence or absence of a test compound. Test compounds able to increase the degradation of the substrate by the protease are identified as potential therapeutic agents useful for the treatment of Alzheimer's disease according to the

5   invention.

For all the above assays utilizing GFP, the GFP can be encoded by its naturally occurring coding sequence or by a coding sequence that has been modified for optimal human codon usage (U.S. Patent No. 5,874,304) when screening in a mammalian cell line. Mutations can be introduced into the coding sequence to produce GFP mutants

10 with altered fluorescence wavelength or intensity or both. Such mutations are largely in the vicinity of residues 65-67, which form the chromophore of the protein. Examples of useful GFP mutations for use as reporter genes according to the methods of the present invention can be found in U.S. Patent Nos. 5,777,079 and 5,804,387 and International Publication WO97/11094. In another preferred mode of the embodiment, the GFP mutant is a Blue

15 GFP. Examples of Blue GFPs are described by Heim & Tsien (1996, *Curr. Biol.* 6:178-82). In yet another preferred mode of the embodiment, the fluorescent protein is a yellow or red-orange emitter recently discovered in reef corals (Matz et al., 1999, *Nature Biotechnol.* 17:969-973).

20   **5.5.   SCREENING FOR THE PRESENCE OF OR**
              PREDISPOSITION TO ALZHEIMER'S DISEASE

The present invention also relates to methods of screening subjects for the presence of or predisposition to Alzheimer's disease. Such a method may comprise measuring activity, level or expression of plasmin, plasminogen, α-enolase or γ-enolase

25 where a reduction in the measured parameter relative to a standard level for the measured parameter (*e.g.*, the level exhibited by a subject not having Alzheimer's disease or a predisposition thereto) indicates the presence of or predisposition to developing Alzheimer's disease. Such a method may also comprise measuring activity, level or expression of PAI-1 or PAI-2 where an increase in the measured parameter relative to a

30 standard level for the measured parameter (*e.g.*, the level exhibited by a subject not having Alzheimer's disease or a predisposition thereto) indicates the presence of or predisposition to developing Alzheimer's disease. The measurements may be performed on any biological sample taken from the subject, preferably blood, plasma, serum, or cerebrospinal fluid.

35

6.   EXAMPLE: IDENTIFICATION OF PLASMIN AS AN APP SECRETASE

In the example presented herein, the identification of plasmin as an APP secretase is demonstrated.

5   ## 6.1   MATERIALS AND METHODS

Cell culture

Cultures of hippocampal neurons were prepared from the brains of 18-d-old rat embryos as described in Goslin and Banker (1991). These neurons survive for several weeks and undergo full polarization when cultured in serum-free medium (N₂) in the

10   presence of a supporting layer of astrocytes. For our experiments cells were kept in culture for 8-15 days (stage 5 neurons).

Raft purification, lipid manipulation and 2D Electrophoresis

Stage 5 neurons were extracted for 1h on ice in buffer A: 1% TritonX-100,

15   25mM MES pH 7.00, 5mM DTT, 2mM EDTA and CLAP (25mg/ml each of chymostatin, leupeptin,antipain and pepstatin A). The extracts were mixed with Optiprep (Nycomed) to reach a final concentration of 40% and overlayered in an SW40 centrifugation tube (Beckman) with a step gradient of 30% and 5% Optiprep in buffer A. After a 5h centrifugation at 35,000rpm in a Beckman ultracentrifuge, the raft fraction was obtained

20   from the interface of the 30% and 5% Optiprep solutions.

In the case of radioactive labelled extracts, cells were incubated with 200µCi/ml 35S methionine for 12 hours prior to the detergent extraction. To identify the true raft proteins, raft lipids were manipulated as follows: sphingomyelin was inhibited by a long term incubation with 25µM Fumonisin B and cholesterol depletion was performed

25   using methyl-β-cyclodextrin as described in Ledesma *et al.*, 1998. Fumonisin and cyclodextrin treated neurons were subsequently radiolabelled, detergent extracted and centrifuged in an optiprep step gradient as indicated above. Samples were analyzed by 2D using the Bio Rad minigel system.

30   *Immunoprecipitation and Western Blots*

Rafts fractions from ³⁵S methionine labeled rat hippocampal neurons were immunoprecipitated in non stringen conditions using 0.5% digitonin (recrystalyzed from Fluka), 3% BSA, 250mM sucrose, 1mM EDTA, 10mM MOPS/KOH pH 7.2 and CLAP for 1h at RT using a monoclonal antibody against the N-terminal of APP (clone 22C11,

35   Boehringer). The immunoprecipitated material was analyzed by 2D electrophoresis and the gels dried and exposed to Kodak X-Omat films.

Non labeled rafts were boiled in Laemmli buffer, loaded in a 10-20%
acrylamide gel (BioRad) and blotted using the previously described polyclonal antibody
against plasminogen. Anti rabbit Ig horseradish peroxidase linked and the ECL method
(Amersham) was used for the detection of the protein.

5   ## 6.2   RESULTS AND DISCUSSION

Raft fractions from mature rat hippocampal neurons were collected from the
interphase 30-5% of an optiprep gradient after the extraction of ^{35}S methionine labeled
cells in 1% TritonX-100 for 1h at 4°C and gradient centrifugation. APP was then
immunoprecipitated from the labeled raft fractions with a monoclonal antibody against the
10 N-terminal domain of the protein in non stringent conditions using digitonin. Figure 1A
shows the autoradiography from the 2D analysis of the immunoprecipitated proteins.
Some peptides were unspecific and were also present in the control raft sample (Figure 1B)
in which equivalent amounts of rafts but only the secondary antibody, protein G sepharose
beads, were used. Among the specific immunoprecipitated proteins several with molecular
15 weights around 50kD were identified (indicated by asterisks and an arrow in the insets 1A,
B).

In order to assign the APP co-immunoprecipitated proteins as raft proteins
we compared the immunoprecipitation profile shown in Figure 1A with that of total raft
components. Thus, we performed 2D analysis of the 30-5% optiprep interphase from
20 radiolabeled Triton X-100 extracted stage 5 neurons. The three spots marked in the inset
of the APP co-immunoprecipitated proteins (Figure 1A) are also present in the total raft 2D
profile (Figure 2A, stage 5), suggesting that they are indeed raft associated proteins.
However, to unequivocally confirm this, radiolabeled mature stage 5 neurons were treated
with inhibitors of two of the main raft lipids; fumonisin, which inhibits the synthesis of
25 sphingolipids, or methyl-β-cyclodextrin, which extracts cholesterol from cell membranes.
The cells were then solubilized in Triton X-100 at 4°C, floated in an optiprep gradient and
the raft fraction (30-5% optiprep) analyzed by 2D electrophoresis. Under these conditions
the three spots disappear from the raft floating material. This is shown in Figure 2A (stage
5 + FB, stage 5+ CD). The figure shows the same areas corresponding to the insets
30 presented in Figure 1 and in Figure 2A (stage 5). The specific raft nature of these three
minor spots was further confirmed when we analyzed the 30-5% optiprep pattern of
membranes obtained from young stage 4 neurons. We have previously shown that these
cells have a deficiency in rafts (Ledesma *et al.*, 1999, *EMBO J.* 18, 1761-1771).
Consistently, we could not detect these spots (Figure 2 B, stage 4). Importantly, the spots
35 were present in the 30-5% optiprep fraction of membranes obtained from stage 4 cells
pretreated with the precursor of sphingolipids, ceramide (Stage 4 +ceramide).

Altogether the data presented above unequivocally confirms that the APP coimmunoprecipitating proteins first identified are true components of the neuronal raft membrane. APP processing is known to occur in this raft fraction (Simons *et al.*, 1998, *Proc. Natl. Acad. Sci. USA* 95, 6460-6464; Lee *et al.*, 1998, *Nat. Med.* 4, 730-734.; Ikezu *et al.*, 1998, *J.Biol.Chem.* 273, 10485-10495).

5   To determine the identity of the three spots behaving as raft proteins and coimmunoprecipitating with APP, we prepared raft material from 100 dishes with cultured hippocampal neurons (150,000 cells/dish), performed the 2D analysis, stained the gel with Coomassie Blue. We were able to obtain enough of the raft APP interacting protein marked in figures 1 and 2 with an arrow to use the MALDI-TOF technique (Nawrocki *et*
10 *al.*, 1998, *Electrophoresis* 19(6):1024-35) to determine the sequence of the visualized spot. The MALDI-TOF analysis identified this protein as the neuronal specific enolase. Neuronal specific enolase participates in the neuronal glycolytic chain and is also present in the neuronal plasma membrane where it is capable of forming dimers with the ubiquitous α-enolase and, in this dimer form, acts as a receptor for the binding of
15 plasminogen to the cell surface (Jorgensen and Centervall, 1982, *J. Neurochem* 39, 537-542; Keller *et al.*, 1994, *J. Neurosci. Res* 38, 493-504; Nakajima *et al.*, 1994, *J. Neurochem*, 63, 2048-2057). Plasminogen binding to the cell surface is a prerequisite for the activation of plasminogen into the proteolytically active plasmin (reviewed in Plow *et al.*, 1995, *FASEB J.* 9, 939-945).

20   The plasminogen system has been linked with extracellular matrix degradation and therefore with processes such as growth and synaptic plasticity in the brain (Seeds et al., 1997, *Cell Tissue Res.* 290, 367-370). Moreover, one of the plasminogen activators, tPA, is one of the early genes activated in long term potentiation and seems to participate in the remodeling of synapses thought to be needed in the acquisition of
25 memory (Qian *et al.*, 1993, *Nature* 361, 453-457; Baranes *et al.*, 1998 *Neuron* 21, 813-825; Madani *et al.*, 1999, *EMBO J.* 18, 3007-3012). Since rafts play a role in APP cleavage, we decided to investigate whether plasminogen activity existed in the neuronal raft fraction, and, if so, the possible role of plasminogen in APP processing.

30 7.   EXAMPLE: LOCALIZATION AND BINDING OF
         PLASMIN/PLASMINOGEN AND α-ENOLASE TO APP

         In the example presented herein, the localization of plasmin/plasminogen and α-enolase to the surface of neurons, and the association of plasmin/plasminogen and α-enolase with APP is demonstrated.

35

7.1   MATERIALS AND METHODS

Immunofluorescence of surface membrane proteins

For the localization of plasmin/plasminogen and α-enolase in the neuronal
surface the cells were incubated with both a monoclonal anti-α-enolase antibody (Quartett,
Biogenesis) and a polyclonal anti-plasmin/plasminogen antibody (Biogenesis) diluted in
5   culture medium for 8 min at 37°C and 5% CO_2. The cells were fixed with 4% PFA and
incubated with rhodamine conjugated anti-mouse IgG from Cappel for the detection of the
anti-α-enolase antibody, or fluoresceine conjugated anti-rabbit from Amersham for the
detection of anti-plasmin/plasminogen antibody. Microscopy was performed with an
Axiophot microscope (Zeiss).
10

Immunoprecipitation and Western Blots

Raft fractions from rat hippocampal neurons were immunoprecipitated in
1% TritonX-100, 50mM NaCl, 2mM EDTA, 10mM Tris pH 7.5 and CLAP at room
temperature using a monoclonal antibody against the N-terminal of APP (clone 22C11,
15   Boehringer). The immunoprecipitated material was loaded in a 10-20% acrylamide gel
and blotted using the previously described antibodies against α-enolase,
plamin/plasminogen or APP. Horseradish peroxidase linked anti-mouse and anti-rabbit
antibodies and the ECL method from Amersham were used for the detection of the
proteins.
20

7.2   RESULTS AND DISCUSSION

To further support the existence of an interaction between plasminogen and
neuronal specific enolase on the neuronal membrane we performed a surface staining of
stage 5 hippocampal neurons using a monoclonal antibody against neuronal enolase
25   (Figure 3Aa) and a polyclonal antibody against plasminogen (Figure 3Ab). White arrows
indicate sites at which both proteins clearly colocalize on the membrane of the neuron.
Figure 3Ac is the phase contrast image of the visualized cell. Both proteins were present
and colocalized to a significant extent (see arrows) on the neuronal surface. Moreover,
Western blot analysis of the neuronal raft fraction with an antibody against plasminogen
30   confirmed its presence in these microdomains. Importantly, not only the 80Kd
plasminogen was present but also the 50Kd and 30Kd fragments that result from
plasminogen activation (Figure 3B). The 30Kd fragment is plasmin, a protein that has
been shown to have proteolytic activity in the brain (Chen and Strickland, 1997, *Cell* 91,
917-925).
35   To determine whether plasminogen activity (plasmin) is exclusive of the
raft fraction or also present in non raft membranes we performed a Western blot with the

same anti-plasminogen antibody in all fractions of our Optiprep gradients. Remarkably, the Western blot analysis revealed that while plasminogen is present in all fractions, the active form of plasminogen, plasmin, is exclusively in rafts (Figure 4). These results confirm the presence of plasminogen in rafts where it is specifically activated to its proteolytic active fragment, plasmin. The hypothesis of a relationship between rafts and proteolysis is, thus, further supported.

5

8.   EXAMPLE: MODULATION OF PLASMIN/PLASMINOGEN ACTIVITY EFFECTS PROCESSING OF APP

In the examples presented herein, it is demonstrated that an inhibitor of plasmin/plasminogen effects the processing of transiently expressed APP.

10

8.1   MATERIALS AND METHODS

Expression of human APP and manipulation of plasminogen activity in mature rat hippocampal neurons

15

Recombinant SFV encoding human APP695 was prepared as described by Olkkonen *et al.*, 1993, *J. Neurosci. Res.* 35:445-451. Stage 5 neurons were infected with SFV-APP for 1.5 hours. Five groups of the transfected neurons were incubated under the following conditions: group a -- 20mM Hepes as a control; group b -- 1 unit of plasminogen; group c -- 1 unit of plasmin; group d -- 40mM Acetyl-Lysine (Sigma) to

20 inhibit the binding of plasminogen to the cells; group e -- 2mg/ml of PAI-1 (American diagnostica), a specific inhibitor of the activation of plasminogen. After another 1.5 hours the medium containing the virus was removed and replaced by labeling medium with 200mCi/ml ^{35}S methionine. All the compounds mentioned above were added to the different groups of cells at the concentrations indicated for the corresponding case. In

25 groups a, b, d and e the same amounts of fresh compounds were added every 1.5 hours during the 5 hour total incubation at 37°C and 5% CO_2. In group c, only 1 unit of plasmin was added after 3h. After the 5h incubation the cells were washed and extracted in 2% Nonidet P-40, 0.2% SDS, 5mM EDTA, 10mM Tris pH 7.2 and CLAP. In order to analyze the fragments produced by the α and β cleavages the samples were immunoprecipitated

30 using the polyclonal antibody B/14 against the C-terminal domain of APP (Simons *et al.*, 1998, *Proc. Natl. Acad. Sci. USA.* 95, 6460-6464) and loaded in Tricine gels (Schagger and von Jagow, 1987, *Anal. Biochem.*166, 368-379) for a better resolution of small peptides. Radioactivity of the individual bands was determined with a PhosphorImager (Molecular Dynamics). The intensity of each band was quantified using the NIH program.

35

The same kind of approach was used to analyze the α-secreted form of APP. Seven day old (stage 5) neurons were infected for 4hr at 37°C and 5% CO_2 with the

recombinant SFV-APP. The medium containing virus was then replaced by fresh N2.

Five groups of the transfected neurons were incubated under the following conditions: group a -- 20mM Hepes as a control; group b -- tPA (American Diagnostica), an activator of the conversion of plasminogen into plasmin, at a final concentration of 0.5 µg/ml; group c -- an equivalent amount of neurons were non infected and treated as in the control.

5   Aliquots from the media were taken after 1h and 2.5h. At this time the cells were washed and extracted in 1%TritonX-100, 0.1%SDS, 100mM NaCl, 20mM tris pH 7.5 and CLAP. The cells and the aliquots from the media were precipitated in 10%TCA, loaded in an 6% acrylamide gel and analyzed by Western blot using the monoclonal antibody 6E10 (Senetek) that specifically recognizes the secreted processed amyloid protein

10 corresponding to the α cut but not the one produced by the β cut.

8.2   RESULTS AND DISCUSSION

In order to analyze the role of plasminogen-plasmin in the proteolysis of APP, stage 5 hippocampal neurons were infected with SFV encoding the human isoform of

15 APP 695 (Simons et al., 1998, Proc. Natl. Acad. Sci. USA. 95, 6460-6464). The overexpressed APP was metabolically labeled with ^{35}S methionine while the cells were treated with different activators or inhibitors of the plasminogen system. These included 1 unit of plasminogen, 1 unit of plasmin, acetyl-lysine (40mM), an inhibitor of the binding of plasminogen to the cells, thereby precluding its conversion to plasmin (Miles et al., 1991,

20 Biochemistry 30, 1682-1691),and PAI-1 (2µg/ml), a specific inhibitor of tPA mediated activation of plasminogen (Baranes et al., 1998, Neuron 21, 813-825). After incubation with these reagents the cells were extracted and immunoprecipitated with an antibody against the C-terminal fragments of APP.

Figure 5A shows the autoradiographs of tricine gels loaded with different

25 samples of ^{35}S methionine labeled stage 5 hippocampal neurons previously infected with SFV encoding the human APP695 isoform. The samples correspond to: non treated infected neurons (control: a,b,c); infected neurons incubated with 1 unit of plasmin (a: plasmin); infected neurons treated with 2mg/ml of PAI-1, an specific inhibitor of the activation of plasminogen, (b: PAI); infected neurons incubated with exogenous

30 plasminogen (b: plasminogen); infected neurons incubated with 40mM acetyl-lysine, an inhibitor of the cellular binding and subsequent activation of plasminogen (c: Lysine). The 12kd fragment corresponding to the β cleavage (b) and the 10kd fragment produced by the α cleavage (a) were immunoprecipitated in all cases using a polyclonal antibody against the C-terminal region of APP.

35 Figure 5B summarizes the results shown in Figure 5A with representative examples. Bars indicate the percentage of the fragment corresponding to the β cut (β) or

the α cut (A) APP with respect to the control. Error bars correspond to the standard
deviation from two different experiments in each case (for lysine, three experiments were
performed). In the activation experiments using plasminogen (Pg) or Plasmin (P) the
amount of β and α fragments were increased. However, the increment was higher for α
cleavage, particularly in the case of addition of plasmin. On the other hand the use of
5   inhibitors of the plasminogen system such as acetyl-lysine (Lys) or PAI-1 (Pai) diminished
the production of β and α fragments by hippocampal neurons in culture. Again the
reduction was mainly observed in the α fragment.

Thus, consistent with the previous results, the use of specific inhibitors of
plasminogen activation (an analog of lysine or the tPA inhibitor PAI-1) resulted in a
10 decrease in the amount of both β and α C-terminal fragments. Again the effects in the α
cleavage were more evident particularly in the case of the lysine competition (in one
experiment this had no effect at all in the β cleavage). To further investigate the role of
plasmin in α-cleavage we analyzed the amount of secreted APP α- fragments. With this
approach we could also avoid the influence that the γ cleavage may have in the recovery of
15 the C-terminal fragments of APP in the cells. Neurons were infected with human APP-
SFV for several hours and the medium replaced with either fresh medium or medium
containing tissue plasminogen activator (tPA). Aliquots of media were taken at different
times and the amount of secreted APP α-fragments (α-sAPP) was analyzed by Western
blot using antibody 6E10, which recognizes a peptide between the α and β cleavage site of
20 APP and is therefore specific for the α-secreted APP form (Ikezu et al., 1998, J.Biol.Chem.
273, 10485-10495). tPA treated cells showed an increased release of α-sAPP at all times
analyzed compared to untreated neurons (data not shown).

The above results demonstrate the role of plasminogen-plasmin in the
proteolysis of APP and suggest that plasmin favors α-cleavage with respect to β-
25 amyloidogenic cleavage.

9.   EXAMPLE: PLASMIN AND tPA INCREASE APP α CLEAVAGE IN
       HIPPOCAMPAL NEURONS OVEREXPRESSING APP

Fully mature hippocampal neurons were infected with Semliki Forest virus
30 (SFV) encoding human APP695. The overexpressed APP was metabolically labeled with
^{35}S methionine while the neurons were incubated in the presence (1 μ/ml) or absence of
plasmin. Cells were then extracted and APP was immunoprecipitated using an antibody
against its twenty carboxyterminal amino acids (Figure 6A). Plasmin addition produced a
consistent 25% increase in the amount of the α-secretase cleaved carboxyterminal fragment
35 with respect to the control. A slight increase (8%) in levels of β-secretase cleaved
carboxyterminal fragment was also observed (Figure 6B).

To further investigate the role of plasmin in α-secretase cleavage, the
secreted APP α-fragments (αsAPP) in the culture medium were assayed. With this
approach a direct measure of the α-secretase is obtained. In the analysis described above
γ-secretase cleavage and lysosomal turnover may affect the recovery of the C-terminal
fragments of APP in the cells. Neurons were infected with human APP-SFV for 3h and the
5   medium replaced with either fresh medium or medium containing a specific activator of
plasminogen or plasmin, tissue plasminogen activator (tPA). Equivalent aliquots of media
were then taken at different times and αsAPP analyzed by Western blot using antibody
6E10, which recognizes an epitope located between the α and β sites of APP, thus being
specific for the α-secretase cleaved form of APP. tPA treated cells showed an increased
10 release of αsAPP at all times analyzed compared to untreated neurons which reached, in
average, 50% increment (Figure 7A). The levels of infection and expression of APP in the
cells were similar (Figure 7B).

The observation that plasmin produced a preferential effect on α cleavage is
in agreement with the fact that α cleavage of APP occurs mainly if not exclusively at the
15 neuronal surface. Other pieces of evidence support the effect of plasmin in α cleavage: a)
plasmin has a natural affinity for Lysine residues, and the α cleavage site in APP is a
Lysine; b) plasmin can activate metalloproteases and all candidate α-secretases, *i.e.*,
ADAM 10, TACE and MDC9 are members of the family of disintegrin metalloproteases.
Interestingly, the activation of ADAMs require cleavage at a sequence RXKX involving a
20 Lysine. Thus, plasmin could cleave APP at the α site directly or through the activation of
other proteases.

**10.   EXAMPLE: PLASMIN AND tPA DECREASE THE LEVELS OF Aβ
        PEPTIDE IN CELLS OVEREXPRESSING HUMAN APP**

25   HEK cells constitutively expressing the human isoform of APP695 were
metabolically labeled in the presence or absence of tPA (0.5μg/ml), the specific activator
for the conversation of plasminogen to plasmin. After a 4h incubation the media were
collected and immunoprecipitated using the monoclonal antibody 4G8 that recognizes Aβ
and p3 peptides. Addition of tPA resulted in a 24% reduction in Aβ compared to the
30 control levels (Figure 8A). Interestingly, p3 release was also reduced but to a lesser extent
(15%). While the reduction in the levels of secreted Aβ can be easily explained by the
increase in α cleavage (Figure 6), a diminution of the amount of p3 could be due to the
degradative effect of residual plasmin activity in the medium. To test this directly, cells
overexpressing human APP were processed as above in the presence or absence of
35 plasmin. The antibody recognizing Aβ and p3 was then used to immunoprecipitate the
released peptides. As show in Figure 8B addition of plasmin to the medium resulted in the

degradation of both Aβ and p3 even at very low concentrations, in the rate of the reported physiological concentration of plasminogen in the serum. One likely interpretation of the above results is that as the α cleavage is enhanced by plasmin, higher amounts of p3 and lower amounts of Aβ will be produced but once in the medium the still active plasmin will degrade not only Aβ but also p3. This is indeed what we observed when plasmin was

5   added directly to the cell medium.

Taken together, these results suggest that brain plasmin affects APP metabolism at different levels: a) by preferentially cleaving at the α site of APP on the neuronal membrane, which in turn would decrease the availability of internalized fragments susceptible for β processing, and b) by degrading released Aβ peptides.

10

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall

15   within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1.   A method of treating or preventing Alzheimer's disease in a subject having or suspected of having Alzheimer's disease comprising administering to the subject a therapeutically or prophylactically effective amount of a molecule that increases the expression of plasminogen, α-enolase, γ-enolase, plasmin or plasmin-like activity.

2.   The method according to claim 1 wherein the molecule is selected from the group consisting of plasmin; plasminogen; chimeric plasminogen; tPA; uPA; chimeric tPA; chimeric uPA; α-enolase and γ-enolase.

3.   The method according to claim 1 wherein the molecule is selected from the group of staphylokinase; aspirin; sulodexide; recombinant brain-derived neurotrophic factor (BDNF); retinoic acid; T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione) and analapril.

4.   The method according to claim 1 wherein the molecule is a peptide able to block active conformation of plasmin inhibitors.

5.   The method according to claim 1 wherein the molecule is an agent able to inhibit expression or release of plasmin inhibitors.

6.   The method according to claim 1 wherein the molecule is transforming growth factor-β.

7.   The method according to claim 1 wherein the molecule is an agent able to enhance expression or release of tPA.

8.   The method according to claim 1 wherein the molecule is an agent able to enhance expression or release of uPA.

9.   The method according to claim 1 wherein the molecule is an agent able to enhance expression or release of plasminogen.

10.   The method according to claim 1 in which the molecule is a nucleic acid molecule comprising a nucleotide sequence which encodes plasminogen, tPA , uPA, γ-enolase or α-enolase.

11.   The method according to claim 10 wherein the molecule is a nucleic acid molecule comprising a nucleotide sequence which encodes plasminogen, and which method further comprises administering plasminogen activator to the subject.

12.   The method according to claim 1 in which the molecule is an antisense nucleic acid molecule comprising a nucleotide sequence which consists of at least 6 contiguous nucleotides complementary to a nucleotide sequence which encodes PAI-1 or PAI-2.

13.   The method according to any one of claims 1-12 wherein the subject is human.

14.   The method according to any one of claims 1-12 wherein the molecule is purified.

15.   A method of screening for a potential therapeutic for Alzheimer's disease comprising:

(a)   co-culturing (i) a cell that is transformed with and expresses a nucleic acid encoding a protein selected from the group consisting of plasminogen; plasmin; and a peptide comprising the plasmin active site and (ii) a cell that is transformed with and expresses a substrate selected from the group consisting of APP; a mutant form of APP having a mutation associated with familial Alzheimer's disease; a fragment of APP comprising the α-secretase site and the β-secretase site; a fragment of a mutant form of APP of having a mutation associated with familial Alzheimer's disease comprising the α-secretase site and the β-secretase site; and a peptide comprising the α-secretase site and the β-secretase site, in the presence of one or more candidate molecules; and

(b)   determining the rate or amount of cleavage at the β-secretase site that occurs, wherein a decrease in said rate or amount relative to the rate or amount in the absence of the candidate molecules indicates that the candidate molecules are a potential therapeutic for Alzheimer's disease.

16.   A method for screening for a compound able to increase the ratio of p3/Aβ produced in the brain of a subject, comprising:

(a)   contacting a test compound with a cell line expressing plasminogen·

and determining the effect of the test compound on plasmin activity
produced by the cell line;

(b)   if the test compound enhances plasmin activity produced by the cell
line, contacting the test compound with a cell line expressing APP
and determining the effect of the test compound on the processing of
APP into p3 and Aβ;

in which test compounds that increase the ratio of p3/Aβ relative to the ratio
detected in untreated cells is identified as a compound able to increase the
ratio of p3 to Aβ.

17.   A method for screening for a compound able to inhibit the
production of Aβ in the brain of a subject, comprising:

(a)   contacting a test compound with a cell line expressing plasminogen
and determining the effect of the test compound on plasmin activity
produced by the cell line;

(b)   if the test compound enhances plasmin activity produced by the cell
line, contacting the test compound with a cell line expressing APP
and determining the effect of the test compound on the production of
Aβ by the cell line;

in which a test compounds that inhibits the production of Aβ relative to the
production of Aβ detected in untreated cells is identified as a compound
able to inhibit production of Aβ.

18.   A method of screening for a potential therapeutic for Alzheimer's
disease comprising determining if a candidate molecule increases the activity, expression
or level of plasminogen, plasmin, α-enolase or γ-enolase in a cell, and identifying a
candidate molecule that increases said activity, expression or level as a potential
therapeutic for Alzheimer's disease.

19.   A method of screening for the presence of Alzheimer's disease or a
predisposition to developing Alzheimer's disease in a subject comprising detecting
decreased activity, expression or level of plasminogen, plasmin, α-enolase or γ-enolase in
the patient and correlating said decreased activity, expression or level of plasminogen,
plasmin, α-enolase or γ-enolase with the presence of Alzheimer's disease or a disposition
thereto.

20.   A pharmaceutical composition comprising an amount of an active

ingredient capable of increasing plasmin-activity in the subject effective to treat or prevent Alzheimer's disease.

21.   The pharmaceutical composition according to claim 20, which further comprises a pharmaceutically acceptable carrier.

22.   The pharmaceutical composition according to claim 20, wherein the active ingredient is selected form the group consisting of plasmin, plasminogen, chimeric plasminogen; tPA; uPA; chimeric tPA; chimeric uPA; α-enolase; staphylokinase; aspirin; sulodexide; recombinant brain-derived neurotrophic factor (BDNF); retinoic acid; T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione); analapril; a peptide able to block active conformation of inhibitors; an agent able to inhibit expression or release of plasmin inhibitors; transforming growth factor-β; an agent able to enhance expression or release of tPA; an agent able to enhance expression or release of uPA; an agent able to enhance expression or release of plasminogen, a nucleic acid encoding plasminogen, tPA, uPA or α-enolase, an antisense nucleic acid complementary to at lease 6 contiguous nucleotides complementary to a nucleotide sequence which encodes PAI-1 or PAI-2.

23.   The pharmaceutical composition according to claim 22, which further comprises a pharmaceutically acceptable carrier.

24.   The pharmaceutical composition according to claim 20, 21, 22 or 23, wherein the composition is formulated for parenteral or intradermal administration.

25.   The pharmaceutical composition according to claim 20, 21, 22 or 23, wherein the composition is formulated for oral administration.

26.   The pharmaceutical composition according to claim 20, 21, 22 or 23, wherein the pharmaceutical composition is formulated as a pill.

27.   The pharmaceutical composition according to claim 20, 21, 22 or 23, wherein the pharmaceutical composition is formulated as a tablet.

28.   The pharmaceutical composition according to claim 20, 21, 22 or 23, wherein the pharmaceutical composition is formulated as a capsule.

29.   The pharmaceutical composition according to claim 20, 21, 22 or 23, wherein the active ingredient is purified.

30.   Use of a composition comprising a molecule that increases the expression of plasminogen, α-enolase, γ-enolase, plasmin or plasmin-like activity for the manufacture of a medicament for the treatment of Alzheimer's disease.

31.   A kit comprising in one or more containers a cell line expressing plasminogen and a cell line expressing amyloid precursor protein, wherein at least one of the cell lines is recombinantly expressing the plasminogen or the amyloid precursor protein.

32.   A kit comprising in one or more containers the cells of claim 15.

33.   A kit comprising a sterile syringe and one or more purified active ingredients selected from the group consisting of plasmin, plasminogen, chimeric plasminogen; tPA; uPA; chimeric tPA; chimeric uPA; α-enolase; staphylokinase; aspirin; sulodexide; recombinant brain-derived neurotrophic factor (BDNF); retinoic acid; T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione); analapril; a peptide able to block active conformation of inhibitors; an agent able to inhibit expression or release of plasmin inhibitors; transforming growth factor-β; an agent able to enhance expression or release of tPA; an agent able to enhance expression or release of uPA; an agent able to enhance expression or release of plasminogen, a nucleic acid encoding plasminogen, tPA, uPA or α-enolase, an antisense nucleic acid complementary to at lease 6 contiguous nucleotides complementary to a nucleotide sequence which encodes PAI-1 or PAI-2.

34.   A method of decreasing the amount of Aβ produced by a cell comprising contacting said cell with a composition capable of increasing the expression of plasminogen, α-enolase, γ-enolase, plasmin or plasmin-like activity in said cell.

35.   The method of claim 34 wherein the amount of p3 produced by the cell is increased.

36.   A method of increasing the degradation of Aβ produced by a cell comprising contacting said cell with a composition capable of increasing the expression of plasminogen, α-enolase, γ-enolase, plasmin or plasmin-like activity in said cell or in the

medium surrounding said cell.

37. A method of screening for a potential therapeutic for Alzheimer's disease comprising:

(a) co-culturing (i) a cell that is transformed with and expresses a nucleic acid encoding a protein selected from the group consisting of plasminogen; plasmin; and a peptide comprising the plasmin active site and (ii) a cell that is transformed with and expresses a substrate selected from the group consisting of APP; a mutant form of APP having a mutation associated with familial Alzheimer's disease; a fragment of APP comprising the plasmin degradation site; a fragment of a mutant form of APP of having a mutation associated with familial Alzheimer's disease comprising the plasmin degradation site; p3; a fragment of p3 comprising the plasmin degradation site; Aβ; a fragment of Aβ comprising the plasmin degradation site; and a peptide comprising the plasmin degradation site, in the presence of one or more candidate molecules; and

(b) determining the rate or amount of degradation of the substrate that occurs, wherein an increase in said rate or amount relative to the rate or amount in the absence of the candidate molecules indicates that the candidate molecules are a potential therapeutic for Alzheimer's disease.

38. The method of claim 37, wherein the substrate is selected from the group consisting of APP; p3; and Aβ.

39. A method for screening for a compound able to increase the degradation of p3 or Aβ produced in the brain of a subject, comprising contacting a test compound with a cell line expressing a substrate selected from the group consisting of APP; a mutant form of APP of having a mutation associated with familial Alzheimer's disease; a fragment of APP comprising the plasmin degradation site; a fragment of a mutant form of APP having a mutation associated with familial Alzheimer's disease comprising the plasmin degradation site; p3; a fragment of p3 comprising the plasmin degradation site; Aβ; a fragment of Aβ comprising the plasmin degradation site; and a peptide comprising the plasmin degradation site, and determining the effect of the test compound on the degradation of the substrate, wherein test compounds that increase the degradation of the substrate relative to the degradation of the substrate detected in

untreated cells is identified as a compound able to increase the degradation of p3 or Aβ.

40.   The method of claim 39, wherein the substrate is selected from the group consisting of APP; p3; and Aβ.

5   41.   A method for screening for a compound able to increase the degradation of p3 or Aβ produced in the brain of a subject, comprising contacting a test compound with

a)   a substrate selected from the group consisting of APP; a mutant form of APP of having a mutation associated with familial

10   Alzheimer's disease; a fragment of APP comprising the plasmin degradation site; a fragment of a mutant form of APP having a mutation associated with familial Alzheimer's disease comprising the plasmin degradation site; p3; a fragment of p3 comprising the plasmin degradation site; Aβ; a fragment of Aβ comprising the

15   plasmin degradation site; and a peptide comprising the plasmin degradation site, and

b)   a protease selected from the group consisting of plasminogen; plasmin; and a peptide comprising the plasmin active site,

and determining the effect of the test compound on the degradation of the substrate,
20 wherein test compounds that increase the degradation of the substrate relative to the degradation of the substrate detected in the absence of the test compound is identified as a compound able to increase the degradation of p3 or Aβ.

42.   A method for screening for a compound able to increase the ratio of
25 p3/Aβ produced in the brain of a subject, comprising contacting a test compound with

a)   a substrate selected from the group consisting of APP; a mutant form of APP of having a mutation associated with familial Alzheimer's disease; a fragment of APP comprising the α and β secretase sites; and a fragment of a mutant form of APP having a

30   mutation associated with familial Alzheimer's disease comprising the α and β secretase sites; and a peptide comprising the the α and β secretase sites, and

b)   a protease selected from the group consisting of plasminogen;. plasmin; and a peptide comprising the plasmin active site,

35 and determining the effect of the test compound on the cleavage of the substrate by the protease at the α and β secretase sites, wherein test compounds that both 1) increase the

cleavage of the substrate at the α secretase site and 2) do not increase the cleavage of the substrate at the β secretase site are identified as compounds able to increase the ratio of p3/Aβ produced in the brain of a subject.

5   43.   A method of increasing plasmin or plasmin-like activity in the brain of a subject in need thereof, comprising administering to the subject a therapeutically or prophylactically effective amount of a molecule that increases the expression of plasminogen, α-enolase, γ-enolase, plasmin or plasmin-like activity.

         44.   The method according to claim 43 wherein the molecule is selected
10 from the group consisting of plasmin; plasminogen; chimeric plasminogen; tPA; uPA; chimeric tPA; chimeric uPA; α-enolase and γ-enolase.

         45.   The method according to claim 43 wherein the molecule is selected from the group of staphylokinase; aspirin; sulodexide; recombinant brain-derived
15 neurotrophic factor (BDNF); retinoic acid; T-686 ((3E,4E)-3-benzylidene-4-(3,4,5-trimethoxy-benylidene)pyrrol idine-2,5-dione) and analapril.

         46.   The method according to claim 43 wherein the molecule is a peptide able to block active conformation of plasmin inhibitors.
20

         47.   The method according to claim 43 wherein the molecule is an agent able to inhibit expression or release of plasmin inhibitors.

         48.   The method according to claim 43 wherein the molecule is
25 transforming growth factor-β.

         49.   The method according to claim 43 wherein the molecule is an agent able to enhance expression or release of tPA.

30   50.   The method according to claim 43 wherein the molecule is an agent able to enhance expression or release of uPA.

         51.   The method according to claim 43 wherein the molecule is an agent able to enhance expression or release of plasminogen.
35

         52.   The method according to claim 43 in which the molecule is a nucleic

acid molecule comprising a nucleotide sequence which encodes plasminogen, tPA·, uPA, γ-enolase or α-enolase.

53.   The method according to claim 52 wherein the molecule is a nucleic acid molecule comprising a nucleotide sequence which encodes plasminogen, and which method further comprises administering plasminogen activator to the subject.

54.   The method according to claim 43 in which the molecule is an antisense nucleic acid molecule comprising a nucleotide sequence which consists of at least 6 contiguous nucleotides complementary to a nucleotide sequence which encodes PAI-1 or PAI-2.

55.   The method according to any one of claims 43-54 wherein the subject is human.

56.   The method according to any one of claims 43-54 wherein the molecule is purified.
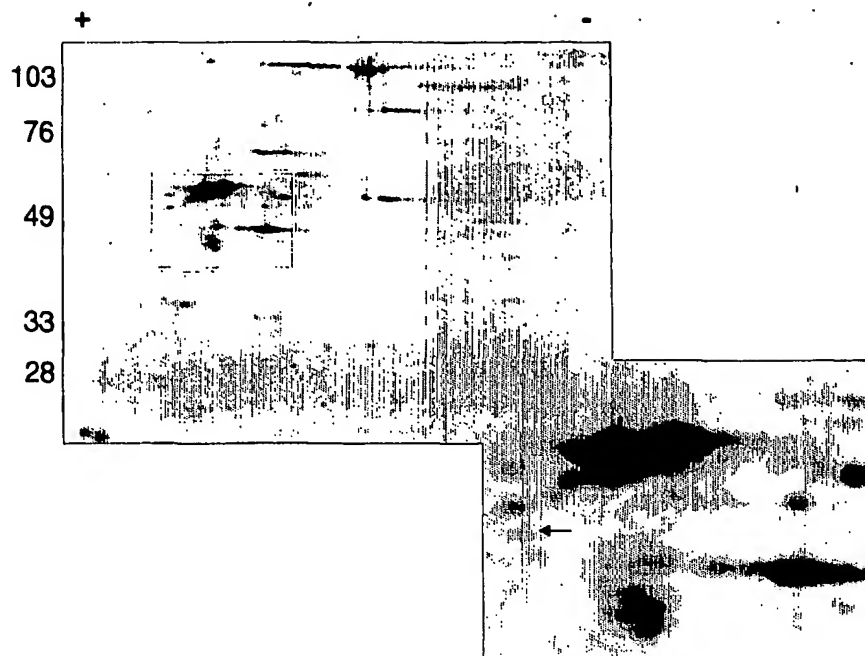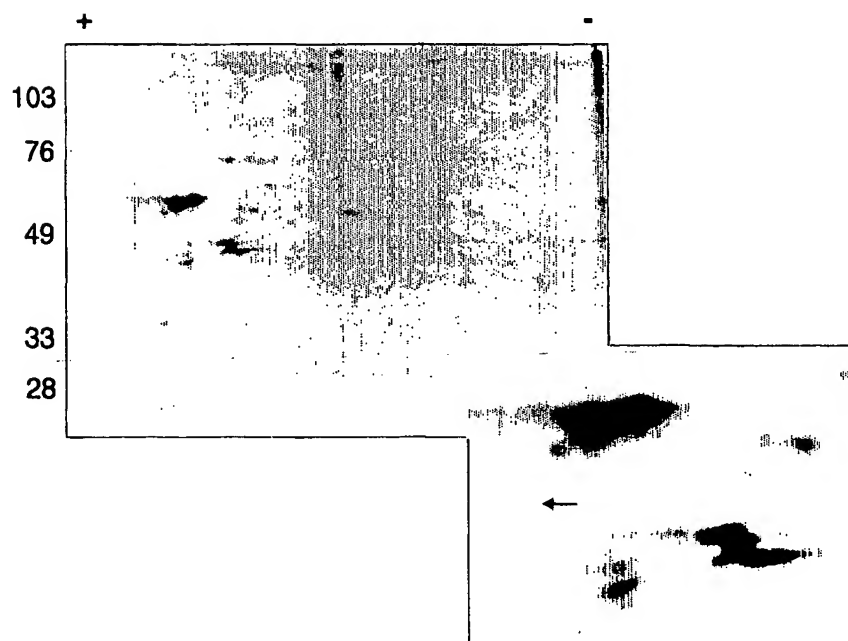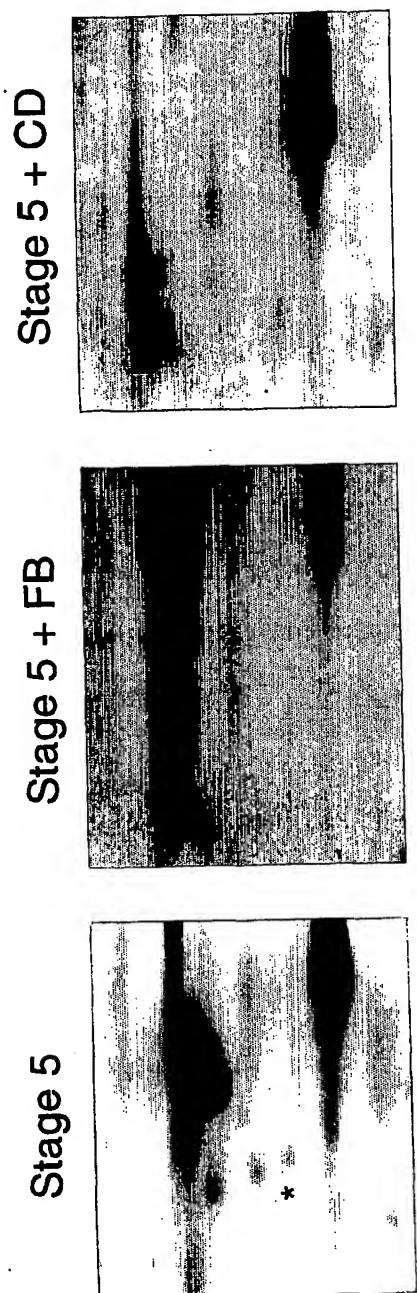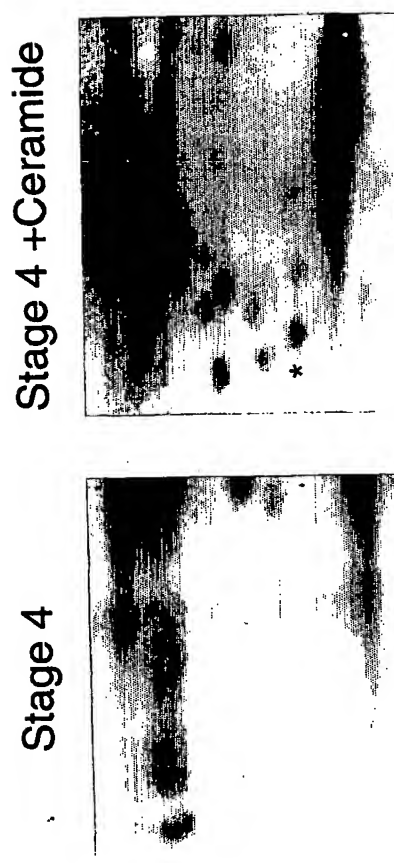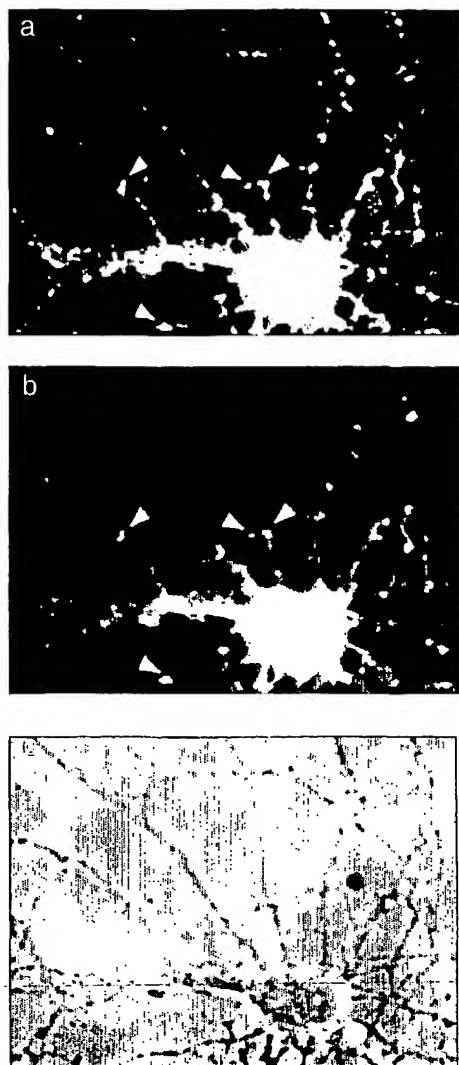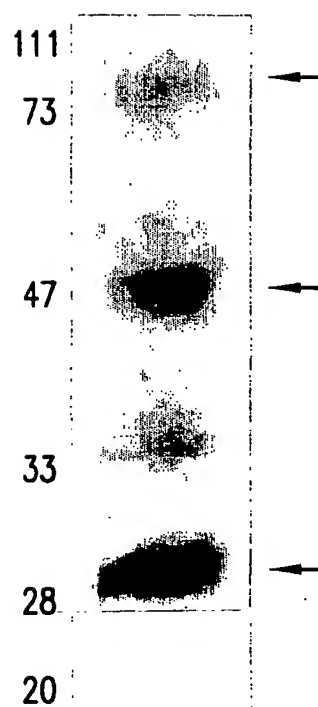
FIG.1A

FIG.1B

FIG.2A

FIG.2B

FIG.3A

FIG.3B

FIG.4

8Kd-

$-\beta$

$-\alpha$

Plasmin   Control

FIG.5A-A

8Kd-

$-\beta$

$-\alpha$

Control   PAI   Plasminogen

FIG.5A-B

8Kd-

$-\beta$

$-\alpha$

Control   Lysine

FIG.5A-C

FIG.5C

FIG.5B

FIG.6A

FIG.6B

5min   1h   2.5h

98kD- | Control

98kD- | tPA

FIG.7A

98kD- | Cont   tPA

FIG.7B

FIG.8

Plasmin (u/ml)   0   0.1   0.01   0.001   0.0001

Aβ –
p3 –
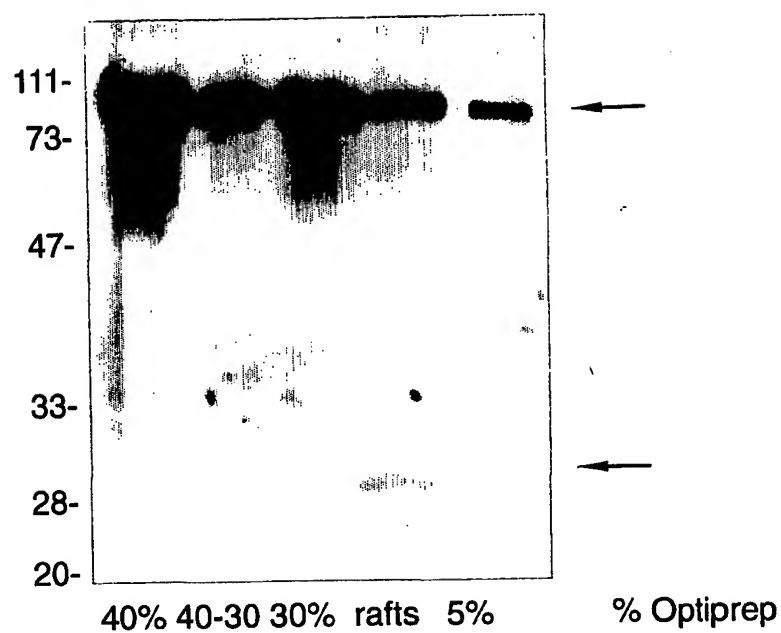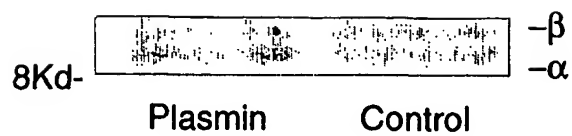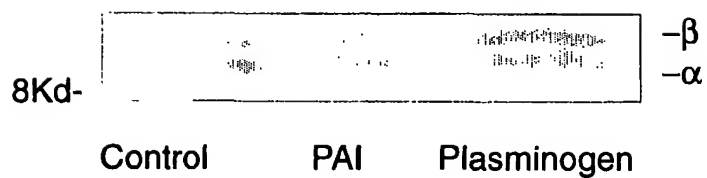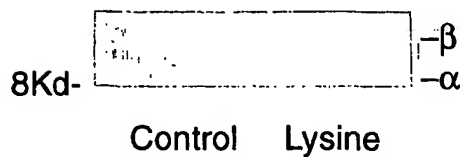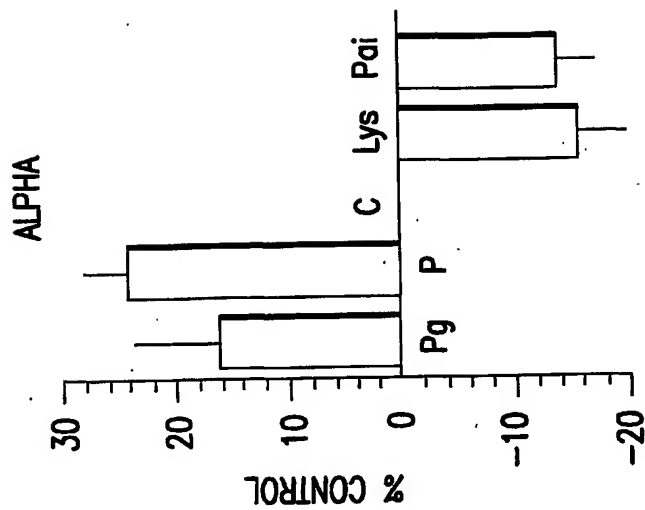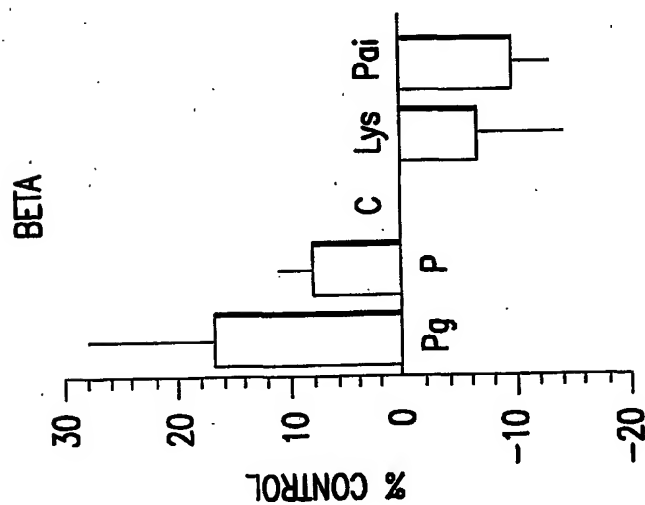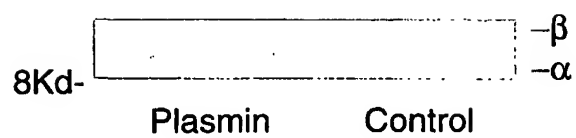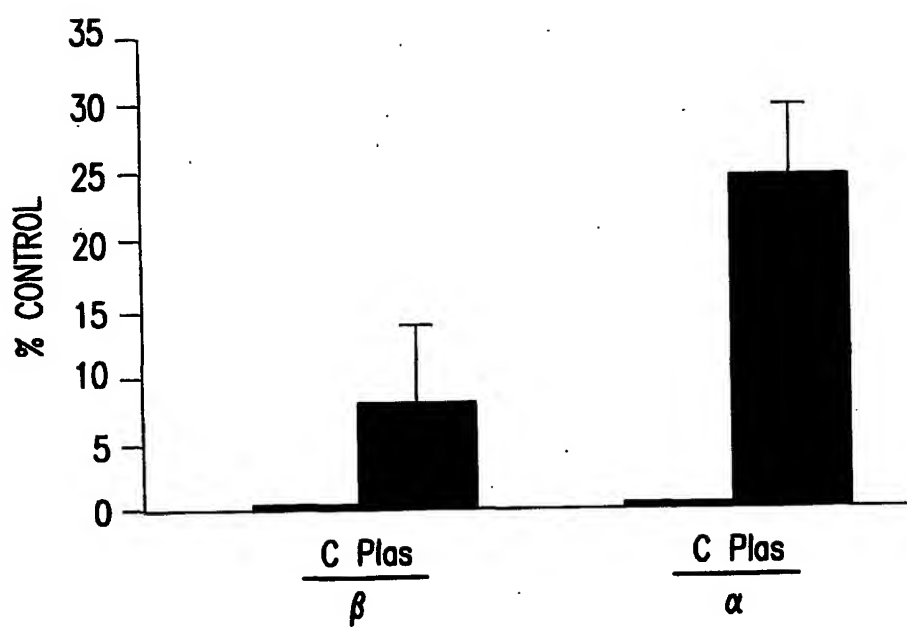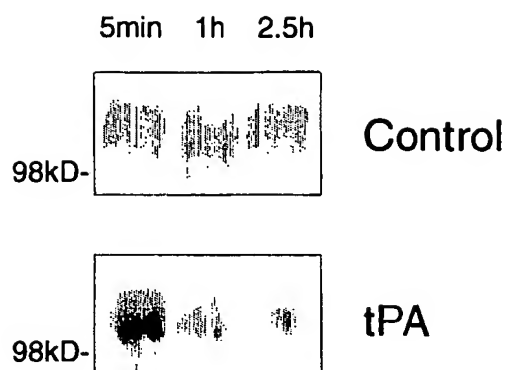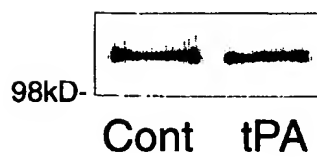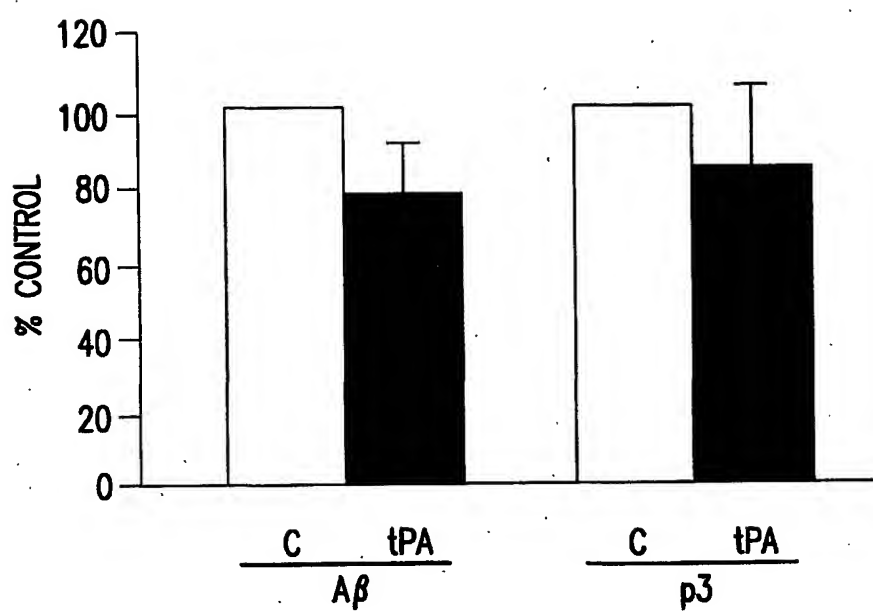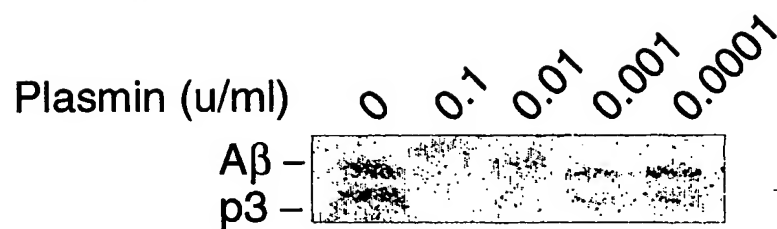
FIG.8B

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